Investigating Flavivirus Infection, Dissemination, and Transmission Dynamics Using Zika virus, West Nile virus, and their Mosquito Vectors

by
Darrell Agbulos, B.Sc.
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Brock University
St. Catharines, ON
L2S 3A1 Canada

Abstract

Flaviviruses (family Flaviviridae, genus *Flavivirus*) are a group viral pathogens responsible for causing disease and death in both humans and animals. Mosquito saliva potentiates *Flavivirus* infection in both *in vitro* and *in vivo* models; however, it remains unknown whether saliva from different species differentially potentiates infection. By inoculating the saliva of different mosquito species plus WNV onto Vero cells, plaque assays were used to study if saliva could differentially potentiate WNV infection. It was found that while there was no significant difference between *Ae. aegypti* and *Ae albopictus* saliva ($p=0.19$), more interestingly was that both saliva treatments had a significant reduction in plaques formed compared to virus alone ($p= 0.01$ and $p=0.00$). The presence of mosquito saliva appears to exert a protective effect *in vitro* when WNV is present.

It also remains to be elucidated as to whether Canadian mosquitoes are able to spread Zika virus. By orally infecting wild caught mosquitoes with a ZIKV infected sugar meal and detecting the presence of virus 10 and 14 days post infection (d.p.i.), the vector competence of Canadian mosquitoes was evaluated. It was found that after 10 ($n=50$) and 14 d.p.i. ($n=32$), 2% and 0% of a population of Culex pipiens mosquitoes were found to be able to become infected and transmit the virus, respectively. Although Culex pipiens mosquitoes from the Niagara region may not be vectors of ZIKV, that does not negate other Canadian mosquitoes as being potential vectors.

Acknowledgements

For my lab, family, and friends.

Thank you for putting up with me during this stressful time in my life.

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List of Abbreviations

Ae. – Aedes

C protein- Capsid protein

CMC- Carboxymethyl cellulose

Cx. – Culex

DC-SIGNR- Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin homologue)

DENV- Dengue virus

DMEM- Dulbecco's Modified Eagle's Medium

D.P.I. – Days post infection

D.R- Dissemination rate

E protein – Envelope protein

EEEV- Eastern equine encephalitis virus

EF1A- Translation elongation factor 1A

ELISA- Enzyme-linked immunosorbent assay

ER- Endoplasmic reticulum

FBS- Fetal bovine serum

I.R- Infection rate

JEV- Japanese Encephalitis Virus

kDa- Kilodalton

M protein- Membrane protein

mosGCTL-1- mosquito galactose specific C-type lectin-1

NCR- Noncoding region

NY99 – WNV New York 1999 strain

ORF- Open reading frame

PBS- Phosphate buffered saline

PFU- Plaque forming unit

prM protein- Pre-membrane protein

PSG- Penicillin streptomycin L-glutamine

qRT-PCR- quantitative reverse transcriptase polymerase chain reaction

RRM- RNA recognition motifs

SDS-PAGE- Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

SGE- Salivary gland extract

Sialome- mRNA and resulting translated proteins expressed in the salivary glands of mosquitoes

siRNA- Small/short interfering RNA

sfRNA- Sub-genomic flavivirus RNA

SL- RNA Stem-loop

SLEV- St. Louis encephalitis virus

TBE- Tick-borne encephalitis virus

T.E- Transmission efficiency

TIAR- T-cell intracellular antigen related

T.R- Transmission rate

VP- Vesicle packet

WNV- West Nile virus

YFV- Yellow Fever virus

ZIKV- Zika virus

Chapter 1

Literature Review

1.1 Biology of *Flaviviruses*

Flaviviruses (family Flaviviridae, genus *Flavivirus*) are a group viral pathogens responsible for causing disease and death in both humans and animals. *Flavivirus* takes its name from the Latin *flavus*, or yellow, the symptom jaundice caused by yellow fever virus (YFV). Other related viruses within the genus include Dengue virus (DENV), West Nile virus (WNV) and Zika virus (ZIKV). Primarily spread by mosquitoes, infected humans can exhibit symptoms ranging from mild flu-like conditions to potentially fatal meningitis, encephalitis, and hemorrhagic fever. Teratogenic effects and neurodegenerative diseases have also been linked to certain flavivirus infections.

1.1.1 Molecular Structure

Mature *Flavivirus* virions are spherical in shape, approximately 50nm in diameter (Murphy, 1980). They are composed of a single stranded, positive sense RNA genome encased by capsid protein. It is housed within a host-derived lipid bilayer that is covered by 180 copies of both envelope and membrane glycoproteins.

1.2 Genome Organization

Nearly 11 Kb long, the 5' capped but 3' non-polyadenylated tail genome encodes a single open reading frame (ORF) that is approximately 3400 codons long (Cleaves and Dubin, 1979). The flanking 5' and 3' -noncoding regions (NCR) of flaviviruses are roughly 100 and 550 nucleotides long, respectively (Wengler *et al*., 1978), depending on strain.

1.2.1 5'-Noncoding Region

The 5' NCR is not translated but plays an integral role in the translation of the ORF. While the 5' NCR sequence varies among viruses within the genus, homologous secondary structures have been observed in both the 5' positive and 3' negative strands. Brinton and Dispoto (1988) described conserved long RNA stem-loop (SL) structures near the terminal end of the 5' NCR of similar size and shape for a variety of flaviviruses and postulated their importance in regulating translation due to conservation of structure despite differences in RNA sequences. Cahour *et al*. (1995) showed that mutant strains with deletions in DENV 5' NCR or in the complementary region of the negative strand, limited translation efficiency *in vitro*. RNA transcripts from the mutant strains reached 40% compared to wild- type virus transcripts. Virulence was also noted to be significantly reduced or removed when mammalian and insect cells were infected *in vitro*. The 3' negative strand has also been implicated in interacting with host factors. Shi *et al*. (1996) found that various cytoplasmic hamster cell proteins bound to the 3’ terminal 75 nt of the WNV minus-strand RNA. Li *et al*. (2002) showed that the high affinity proteins were TIAR (T-cell intracellular antigen related), RNA binding proteins with RNA recognition motifs (RRM). It was also found that WNV replication in a TIAR knockout murine cell line was reduced highlighting the importance of NCRs interacting with host factors.

1.2.2 3'- Noncoding Region

Like the 5' NCR, the 3' NCR shows sequence variation among viruses but shared homologous secondary structures such as the long RNA SL (Grun *et al*., 1986). It contains 3 domain regions, Domain I is a hypervariable region that is followed by two stem loop domains (Wang *et al*., 1996). Domain II assumes a dumbbell (Silva *et al*., 2008) structure while Domain

III contains a short hairpin and stem loop elements (Davis *et al*., 2013; Sztuba-Solinska *et al*., 2013). Studies by Zeng *et al*. (1998) showed that mutant strains of DENV with deletions or substitutions involving a 3' NCR SL resulted in reduced or abolished virulence when inoculated in mammalian or insect cells. Within the study, it was noted that one mutant with a 7 bp substitution within the SL region grew well in LLC-MK2, monkey kidney cells, but saw reduced amplification in a C6/36 mosquito cell line suggesting an interaction with host factors. Blackwell and Brinton (1997) showed through UV cross-linking and gel mobility shift assays that WNV RNA not only bound hamster cell proteins to the 5' NCR SL but also in the 3' SL. Subsequent purification identified one of the proteins as translation elongation factor 1A (EF1A) (Blackwell and Brinton, 1997), a protein known to transport tRNA to ribosomes suggesting its role as a host factor that influences translation. The presence of subgenomic flavivirus RNA (sfRNA) is also dependent on the 3' NCR. These small molecules (300-500 nt) are the product of incomplete digestion by host cell nucleases that disassociate on the 3'NCR (Pijlman *et al*., 2008). Full length sfRNAs have been shown to increase virulence in mice (Liu *et al*., 2014) inhibit antiviral immune responses such interferon (Schuessler *et al*., 2012) and Dicer (Schnettler *et al*., 2012) and inhibit host cell nuclease Xm1, preventing viral genome digestion (Moon *et al*., 2012).

1.2.3 Viral Proteins

When translated, the single ORF yields one large continuous polyprotein that is later spliced into 10 smaller proteins. The N-terminal region of the viral genome encodes 3 structural proteins: the capsid (C), membrane (M), which is first translated as premembrane (prM), and envelope (E) (Rice *et al*., 1985). The remaining region within the ORF encodes the other 7 non-

structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, which regulate and assist replication (Rice *et al*., 1985).

1.2.4 Structural Proteins

1.2.5 Capsid Protein

Capsid is a small, very basic protein (~11kDa) expressed with 114 amino acids (aa), which is translocated to and inserted into the endoplasmic reticulum (ER) via a C-terminus signal sequence. In the ER lumen, cellular signal peptidase cleaves the signal sequence freeing the rest of the polyprotein while the N- terminus end of the signal sequence located on the cytoplasmic side is later cleaved by viral proteases NS2B/3 thus releasing mature C protein (Amberg *et al*., 1994; Boege *et al.*, 1983; Trent, 1977). C protein has 4 alpha-helices linked by short loops which coalesce to form 2 conserved segments: a hydrophobic and a cationic region respectively (Markoff *et al*., 1997), later visualized through NMR and X-ray crystallography (Dokland *et al*., 2004; Ma *et al*., 2004). Known to encapsidate the viral genome, it is assumed that the C-terminus alpha4- alpha4' region associates with viral RNA while the hydrophobic core within the alpha2-alpha2' region associates with both viral and host lipid membranes (Markoff *et al*., 1997). Kofler *et al*. (2002) highlighted the structural importance of C protein using mutant strains of tick-borne encephalitis virus (TBE). Strains with deletions corresponding to the internal hydrophobic domain yielded a decrease in TBE virions *in vitro* and an increase in the formation of attenuated yet immunogenic subviral particles which closely resembled recombinant subviral particles (RSP), virions with E and M protein expressed only. Interestingly non- traditional roles have been found. Known to be translocated to the ER, C protein was also discovered to accumulate

specifically within the nucleolus and has been implicated in mediating apoptosis (Bhuvanakantham *et al*., 2010; Bulich & Aaskov, 1992; Westaway *et al*., 1997). Samuel *et al.* (2016) discovered a novel function in the mosquito host showing that C protein has RNA silencing properties effectively limiting an immune response in *Aedes aegypti* (Linnaeus) mosquitoes, making it easier for flaviviruses to establish infection.

1.2.6 Membrane Protein

M protein (~10 kDa) is initially expressed as the 165 aa long, pre-membrane protein (prM) that is glycosylated. It later undergoes cleavage from cellular furin protease, releasing the pr peptide from the now mature 77 aa long M (Stadler *et al*., 1997). Translocated to the ER lumen via an N- terminus signal sequence, two C-terminus transmembrane domains (TMD) anchor prM to the ER membrane (Hsieh *et al*., 2011). The anchor closest to the C-terminus end acts as the signal sequence for the connected E protein, where it too is translocated to the ER lumen. prM is glycosylated in the ER lumen but awaits further maturation as the assembling virion traverses through the Golgi network (MacKenzie & Westaway, 2001). Located near the N- terminus region are 1-3 glycosylation sites embedded within 2 beta-sheets linked by disulfide bridges (Nowak and Wengler, 1987). prM and E interact via a heterodimeric complex post polyprotein synthesis (Lorenz *et al*., 2002), revealing the chaperone role prM assumes in regulating E folding (Konishi and Mason, 1993; (Zheng *et al*., 2014). Li *et al*. (2008) later visualized the interaction capturing pr peptide in complex with an important ectodomain of E. *Flavivirus* prM associates with E due to it being closely positioned distal to E domain II (DII) inside the heterodimer, sterically hindering E fusion-loop (Zhang *et al*., 2003), preventing premature viral fusion within the cell (Yu *et al*., 2009).

1.2.7 Envelope protein

E protein (~53 kDa) is expressed as a 495 aa protein that is glycosylated and that coats the outermost layer of the virion. It is responsible for mediating binding to host cellular membranes thus prompting fusion. It is composed of 3 domains: Domain I which contains the N-terminus and assumes a beta barrel configuration (Rey *et al*., 1995); Domain II is comprised of two finger-like structures and contains a hydrophobic fusion loop that mediates insertion into the host cellular membrane (Allison *et al*., 2001); Domain III is an immunoglobulin-like domain thought to interact with and bind to cellular receptors (Rey *et al*., 1995). Virion fusion and maturation require structural rotations between all 3 domains (Bressanelli *et al*., 2004; Modis *et al*., 2004)

1.2.8 Non-Structural Proteins

1.2.9 NS1

NS1 (~46 kDa) is embedded into the lumen of the ER after cleavage from the rest of the polyprotein at its N-terminus by host signal peptidase and an unknown enzyme at the C-terminus (Chambers *et al*., 1990; Falgout *et al*.,1989). Found within cells, it has also been located on the cell surface, as well as being secreted from mammalian cells (Mason, 1989; Post *et al*., 1991; Smith *et al*., 1970; Winkler *et al*., 1988). Within the cell, NS1 is thought to aid in RNA replication as it has been observed localizing to vesicle packets (VP), produced from the ER, along with other viral proteins, where the replication complex can be assembled (Mackenzie *et al*., 1998). Mutant YFV strains with changes in NS1 glycosylation sites reduced rates of RNA replication, virus propagation and virulence (Muylaert *et al*., 1996). Specifically, NS1 seems to

be involved in early RNA replication. Lindenbach and Rice (1997), found that a YFV mutant with a large deletion within the NS1 gene produced undetectable amounts of RNA as determined by sensitive assays for detecting the first cycle of minus strand synthesis. NS1 plays an integral role in the formation and/or function of host ribosomal proteins where it is able to localize the subunits at sites of viral replication (Cervantes-Salazar *et al*., 2015). In the presence of siRNA, decreases in viral translation, replication and virion production were noted (Cervantes-Salazar *et al*., 2015). Extracellular activity of NS1 was first postulated to modulate host immune response to facilitate infection (Chang *et al*., 2002; Falconar, 1997), and was later discovered by Kurosu *et al*. (2007) to associate with complement system proteins, inhibiting viral recognition of infected cells. Another form of NS1, NS1' also exists both inter and extracellularly and is the result of alternative splicing. (Melian *et al*., 2010). It has been found to associate with NS3 and NS5 replication complexes (Takamatsu *et al*., 2014), as well as localizing with NS1 where it can offer substituted function (Young *et al*., 2013).

1.2.11 NS2A

NS2 (~22 kDa) is a hydrophobic protein that spans the ER membrane. Cleaved at the N-terminus region by an unknown enzyme while the C-terminus is cleaved by viral serine protease in the cytoplasm (Falgout and Markoff 1995). Found to bind with NS3 and NS5 proteins, 3'NCR sequences, as well as localizing to VPs (Mackenzie *et al*., 1998), NS2A has been implicated in mediating RNA replication, virion formation, viral dissemination as well as regulating host immune responses (Leung *et al*., 2008; Liu *et al*., 2006; McElroy *et al*., 2006; Rossi *et al*., 2007).

1.2.12 NS2B

NS2B (~14 kDa) is a membrane spanning protein that associates and forms a complex with NS3 protein thus being a cofactor for the serine protease activity in NS3 which is required for the cleavage of the polyprotein (Falgout *et al*., 1991). NS3 does not fold properly without interaction with NS2B and thus has no activity (Kim *et al*., 2013). Studies using strains with mutations disrupting the NS2B-NS3 complex removed serine protease activity (Chambers *et al*., 1993; Jan *et al*., 1995).

1.2.13 NS3

NS3 (~70 kDa) plays an integral role in a variety of functions including RNA replication and polyprotein cleavage. Known to associate with NS2B (Falgout *et al*., 1991), it has also been found to localize to VPs and convoluted membranes (CM), sites of polyprotein processing (Westaway *et al*. 1997). Protease activity of NS3 was located to the N-terminal third of the protein, being predicted first by Gorbalenya *et al*. (1989a) and later proven by the observation of strains with mutations within the catalytic triad that saw reduced serine protease activity (Chambers *et al*., 1990). It also has been found to exert protease activity on host cell proteins that would try to prevent infection (Yu *et al*., 2012). Located within the C-terminus region are encoding regions for helicases, helping unwind RNA duplexes (Gorbalenya *et al*. 1989b), where it was later found to have helicase, nucleoside 5'-triphosphatase (NTPase), and RNA 5'-triphosphatase (RTPase) activity (Li *et al*., 1999).

1.2.14 NS4A and NS4B

NS4A and NS4B (16 and 27 kDa respectively) are hydrophobic proteins that have have been implicated in mediating RNA replication as they have been observed to localize to both VPs and CMs as well as directly interacting with NS1 (Mackenzie *et al*., 1998; The C-terminus of NS4A contains a signal sequence that translocates NS4B to the ER lumen. NS1 and NS4A/B complexes can associate with complement system proteins to counteract immune responses (Muller and Young, 2013).

1.2.15 NS5

NS5 (103 kDa) is a highly conserved, large protein implicated in capping, methylation, and replication of the genome (Dong *et al*., 2012; Egloff *et al*., 2007). It consists of an N-terminus methyltransferase and a C-terminus RNA- dependent-RNA polymerase domain (Ackermann and Padmanabhan 2001; Guyatt *et al*., 2001; Nomaguchi *et al*., 2004).

1.3 Life Cycle

The life cycle of WNV is well studied and will serve as the model for flavivirus infection and replication. WNV virions collide with the surfaces of cells gaining entrance only by associating with target cellular surface receptors. Many cellular surface proteins have been implicated as the receptor for West Nile virus; however, two of the most studied and likely candidates include mammalian C-type lectin receptor DC-SIGNR (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin homologue) and mosquito galactose specific C-type lectin-1 (mosGCTL-1). DC-SIGNR is thought to interact with glycosylated prM and E proteins of the WNV virion (Davis *et al*., 2006). WNV is still able to bind to cells without

DC-SIGNR suggesting a wider variety of receptors (Davis *et al*., 2006). Mosquito cellular receptor mosGCTL-1 was found to be able to bind WNV enabling attachment and enhanced entry into mosquito cells (Cheng *et al*. 2010). After binding to a receptor, the virion enters the cell through a process known as receptor mediated clathrin dependent endocytosis. The virion enters as the plasma membrane and invaginates around to form an endosome. This vesicle containing the virus is formed with the protein clathrin. The triskelion shaped clathrin and its accessory proteins polymerize around the vesicle mediating entry into the cell. WNV is thought to follow this mode of entry as studies using compounds specifically inhibiting clathrin dependent endocytosis greatly inhibited infection (Chu *et al*., 2006). As the endosome enters the cell a decrease in pH causes the virion E protein to undergo a conformational change, allowing it to fuse with the perimeter of the endosome. This fusion event allows the viral genome to be released from the endosome and into the cytoplasm of the cell (Brinton 2002). The viral mRNA associates with ribosomes on the ER where it is translated into a polyprotein. This polyprotein is then processed within the endoplasmic reticulum where it is cleaved by cellular proteinases resulting in 3 structural and 7 non-structural proteins. The 7 non-structural proteins mediate the replication of viral RNA through the formation of an RNA replicase complex. These complexes associate with the viral genome creating more copies used to further replication and associate with the structural proteins when packaging new virions. The virions are assembled as they traverse through the Golgi apparatus where upon maturation, they are exit from the cell through exocytosis. After the virus leaves the cell, it is free to interact with adjacent cells, continuing the cycle (Brinton 2002).

1.4 West Nile Virus

In 1999, New York City health officials noticed an increase of patients afflicted with meningoencephalitis (CDC, 1999). Around the same time, local crow populations were seeing higher fatalities with zoos reporting suspicious deaths of exotic avian species also afflicted with meningoencephalitis (CDC, 1999). Sera isolations from both human and avian cases confirmed through PCR and ELISA that for the first time WNV had been found in the Western hemisphere. By the end of the year, 59 patients were hospitalized as well as 7 deaths recorded due to complications caused by the WNV NY-99 strain (Nash, *et al*., 2001). Its eventual spread throughout North America while causing numerous more outbreaks has resulted in WNV being labeled as the leading cause of mosquito-borne/ epidemic encephalitis in the United States (CDC, 2012).

West Nile Virus (WNV; family Flaviviridae, genus *Flavivirus*) is an enveloped, positive-sense, single-stranded RNA virus. Approximately 11 kb long, it contains all the elements of a generic flavivirus as well as having a similar replication cycle. Originating in Africa, WNV has slowly spread throughout Europe, Asia, and the Americas causing death and neurotropic disease including meningitis and encephalitis.

1.4.1 History

WNV was first isolated in 1937 from the serum sample of a febrile patient residing in the West Nile district of Uganda (Smithburn *et al*., 1940). It was later determined to be a new neurotropic pathogen like other viruses belonging to the Japanese Encephalitis complex based on serological cross-reactivity (Smithburn, 1942). Philip and Smadel (1943) showed that WNV was most likely spread by infected mosquitoes with Work *et al*. (1955) discovering that birds acted as

reservoir hosts in an enzootic cycle with mosquitoes. Up until 1975, WNV fever epidemics in Israel and South Africa and a WNV meningoencephalitis outbreak in Europe were noted (Klingberg *et al*., 1959; Murgue *et al*., 2001; McIntosh *et al*., 1976). Unreported until 1994, large epidemics started to occur in Africa, Europe and the Middle East ultimately culminating in the surprising arrival of WNV in the Western hemisphere (Campbell *et al*., 2001; Murgue *et al*., 2001).

1.4.2 Phylogeny

Different strains of WNV can belong to one of two lineages (Lineage I and II) (Berthet *et al*., 1997). Lineage I strains of WNV are found globally including NY-99, most commonly associated with causing the New York outbreak of 1999 (Berthet *et al*., 1997; Lanciotti *et al*., 1999). Differences in pathogenicity of strains have been noted within Lineage I (Brault *et al*., 2007). Lineage II strains of WNV are endemic to sub-Saharan Africa and parts of Europe (Bakonyi *et al*., 2005). Lineage II strains of WNV have been associated with a reduced severity of disease and sporadic cases of neuroinvasion. Interestingly WNV NY-99 had been determined to be most closely related to a WNV isolate from Israel in 1998 (Lanciotti *et al*., 1999), although no explanation has been found for its arrival in the United States.

1.4.3 Transmission cycle

In nature, WNV is propagated in an enzootic cycle between mosquitoes, predominantly from the genus *Culex,* and birds (Kilpatrick *et al*., 2005; Work *et al*., 1955). Mammals are generally considered to act as dead-end hosts as WNV never attains a sufficient viremia to be transmitted to feeding mosquitoes (Austgen *et al*., 2004; Bunning *et al*., 2002; Xiao *et al*., 2001).

While birds play an integral role in propagating WNV, certain avian species have been implicated in helping it spread. Common birds such as the American crow (*Corvus brachyrhynchos*), blue jay (*Cyanocitta cristata*) and house sparrow (*Passer domesticus*) develop high levels of viremia, increasing the likelihood of infecting feeding mosquitoes (Brault *et al*., 2004; Komar *et al*., 2003; Langevin *et al*., 2005 Weingartl *et al*., 2004). Both mammalian and avian species that have been infected can exhibit symptoms of disease, but most commonly become asymptomatic carriers. Uninfected mosquitoes that feed on infected hosts can become infected depending on a variety of factors. One of the most important is the concentration of virus. Turell *et al*. (2002) found that the minimum titer of WNV needed to infect different mosquito species was between 10^4 - 10^5 plaque forming units/ mL.

1.5 Zika Virus

In 2014, numerous cases of patients with rash, fever and arthralgia were being reported in Brazil. Thought to be an outbreak of DENV or Chikungunya virus, it was later determined through qRT-PCR and ELISA that the culprit was ZIKV (Campos *et al*., 2015; Zanluca *et al.*, 2015). Although previously found in other parts of the world, the outbreak in Brazil marked the first time ZIKV had ever been found in the Western hemisphere. Worryingly, increased reports of microcephaly in newborns and neurodegenerative disease implicated ZIKV as being the causative agent. Spreading throughout the Americas and causing outbreaks, ZIKA has spurred research efforts in combatting both mosquitoes and virus to curb the spread of disease.

Zika virus (ZIKV; family Flaviviridae, genus *Flavivirus*) is an enveloped, positive-sense, single-stranded RNA virus Like WNV, it is ~11kb long and contains similar genetic elements.

Originating in Africa, ZIKV has slowly spread throughout Asia, Oceana, and the Americas and has been implicated in inducing teratogenic effects and causing neurodegenerative disease.

1.5.1 History

ZIKV was first discovered in the Zika Forest of Uganda in 1947 from the blood of a febrile Sentinel Rhesus monkey (Dick, 1952; Dick *et al*., 1952). In 1948, it was then found in pools of *Aedes africanus* (Theobald) mosquitoes (Dick *et al*., 1952). Macnamara (1954) then found the first case of human infection by testing the sera sample of a febrile girl from Nigeria. Spreading out of Africa, ZIKV was detected in Asia for the first time when pools of *Ae. aegypti* mosquitoes collected from Malaysia tested positive for the virus (Marchette *et al*., 1969). The first human cases in Asia were then found in Indonesia (Olson *et al*., 1981). Until 2007, ZIKV only manifested itself through sporadic individual cases. Outbreaks in Gabon and Yap Island prompted investigation which revealed large populations of people testing positive for previous infection of ZIKV as well as implicating both *Aedes hensilli* (Farner) and *Aedes albopictus* (Skuse) mosquitoes as potential vectors (Duffy *et al*., 2009; Grard *et al*., 2014). An outbreak later occurred in French Polynesia where it has been estimated that 28,000 people were infected (Musso *et al*., 2014). Increased cases of Guillaine-Barre syndrome also seemed to coincide with the ZIKV outbreak. Finally, in 2014, ZIKV arrived in the Western hemisphere, first being detected in Brazil (Campos *et al*., 2015; Zanluca *et al*., 2015) before spreading throughout the Americas. Reports of increased cases of microcephaly in Brazil during the ZIKV outbreak (Cao-Lormeau *et al*., 2016) prompted great concern from neighboring countries anticipating its arrival.

1.5.2 Phylogeny

ZIKV has been assumed to belong to one of two lineages, African and Asian/American (Weaver *et al*. 2016). African strains of ZIKV can further be categorized into two groups. Group one or the Uganda cluster is predicated on the MR766 strain first found in Uganda (Dick *et al*., 1952), which also includes strains from Senegal and Central African Republic (Weaver *et al*. 2016). Group two or the Nigeria cluster includes strains isolated from both Nigerian and Senegal from 1968 to 1997 (Weaver *et al*. 2016). The Asian cluster is predicated on the P6-740 strain found in Malaysia (Marchette *et al*., 1969) and includes strains from Micronesia and French Polynesia (Oehler *et al*., 2014). Strains circulating throughout the Americas are assumed to be derived from an Asian strain based on comparative genomic studies (Enfissi *et al*., 2016).

1.5.3 Transmission Cycle

How ZIKV is propagated in nature is debated. It has been assumed that ZIKV is maintained in an enzootic cycle between *Aedes* mosquitoes and an unknown nonhuman primate. Multiple studies since the discovery of ZIKV have implicated *Aedes* mosquitoes as being probable vectors such as the discovery of ZIKV positive wild caught mosquitoes, successful vector competence studies, as well their general abundance and previous history in spreading other flaviviruses. (Aliota *et al*., 2016; Amraoui *et al*., 2016; Boccolini *et al*., 2016; Dick, 1952; Duffy *et al*., 2009; Dick, 1952; Duffy *et al*., 2009; Grard *et al*., 2014; Grard *et al*., 2014; Guedes *et al*., 2016, Guo *et al*., 2016; Huang *et al*., 2016; Macnamara, 1954; Marchette *et al*., 1969; Weger-Lucarelli *et al*., 2016). The assumption of a nonhuman primate is based on the reasoning that it was first discovered from a Sentinel Rhesus monkey (Dick, 1952; Dick *et al*., 1952); however, those studies have postulated that nonhuman primates may not be the most suitable

reservoir hosts as it was rarely found in them. The transmission cycle requires further investigation.

1.6 Implicated Vectors

1.6.1 *Culex* mosquitoes

Species of mosquitoes belonging to the *Culex pipiens* species complex are thought to be primary vectors of WNV in North America due to their proliferation in urbanized areas (Vinogradova, 2000), the coinciding of outbreaks and *Culex* population peaks (Spielman, 2001), wild populations testing positive for WNV (Bernard *et al*., 2001), females being able to transovarially transmit the virus (Dohm *et al*., 2002) and their affinity for avian blood meals (Kilpatrick *et al*., 2005; Work *et al*., 1955). The two species most implicated in WNV transmission are *Culex pipiens* (Linnaeus) and *Culex quinquefasciatus* (Say), the northern and southern house mosquitoes, respectively. Their ability to survive in both temperate and tropical regions as well as their population distribution closely mirroring human activity makes them suitable candidates for transmitting WNV. While *Culex* mosquitoes are most commonly associated with WNV, they can also act as suitable vectors for a variety of other related diseases such as Japanese Encephalitis virus (JEV) and even ZIKV (Guedes *et al*., 2016, Guo *et al.*, 2016; Takahashi, 1976).

It is theorized that ancestral *Culex pipiens* derived from an African species that eventually spread throughout Europe before arriving in the New World possibly in the 16th century (Vinogradova, 2000). It has been argued that their success was due to appropriating food and nutrients found in standing water left by humans and animals (Vinogradova, 2000).

Interestingly within the complex is a subspecies that does not require blood to mature a batch of eggs. *Cx. pipiens* form *molestus* exhibits autogenous behaviour, using only the nutrients and energy sequestered during the larval stage to develop its eggs. *Cx. pipiens* form *molestus* is thought to be a form of the complex that had adapted to survive cold European winters by surviving in manmade underground systems (Fonesca *et al*., 2004, Spielman *et al.*, 2001).

1.6.2 *Aedes* Mosquitoes

Aedes spp. mosquitoes, specifically from the subgenus Stegomyia, are largely responsible for spreading a variety of diseases throughout both the developed and undeveloped world. Known to transmit dengue, yellow fever, chikungunya, and Zika virus amongst other pathogens, they have evolved to associate closely with humans where they facilitate the spreading of these diseases. Their ability to lay eggs resistant to desiccation combined with their preference to lay eggs in artificial containers has facilitated the transport and spread of invasive species to new habitats, (Lounibos and Kramer, 2016; Powell and Tabachnick, 2013). Two of the most important disease vectors *Ae. aegypti* and *Ae. albopictus* have been implicated in helping spread numerous diseases due to their preference for human blood as well as being competent vectors for multiple arboviruses (Ponlawat and Harrington, 2005).

Ae. aegypti has been theorized to have originated in sub-Saharan Africa with the ancestor most resembling the present day *Ae. aegypti* form *formusus* (Tabachnick, 1991). It has been speculated that increasing drought conditions forced the ancestor to domesticate in water laden human settlements (Mattingly, 1967). *Ae. albopictus* has been theorized to have originated in the forests of Southeast Asia and fed on local wildlife (Paupy *et al.*, 2009). Like *Ae. aegypti*, changes

in the surrounding environment led to the domestication of *Ae. albopictus* feeding on human populations.

1.7 Vector Competency

For a mosquito to be considered a competent vector of an arbovirus, it must adhere to a set of rules proposed by Hardy *et al*. (1983): a primary infection of the midgut; virus disseminates throughout midgut epithelium; virus disseminates from the midgut to surrounding tissue; propagation of virus outside of midgut; infection of salivary glands; and the eventual transmission of virus through salivary secretions. Simply, virus must infect the midgut, disseminate throughout the body and finally infect the salivary glands where it can be secreted. While there are many factors that can influence vector competency such as intrinsic/genetic, behavioral, or even geographical, two important barriers to mosquito host infection are the midgut and salivary gland barriers.

1.7.1 Midgut Barriers

The newly acquired viremic bloodmeal is quickly drawn into the midgut where the bloodmeal is to be processed for digestion. Virions enter the midgut lumen where they must attach to and infect the midgut epithelial cells. The process is time sensitive as virions must fuse with and enter midgut epithelial cells before the peritrophic matrix is secreted (Franz *et al.*, 2015). The peritrophic matric is a chitinous sac that can prevent virions in the gut lumen from interacting directly with epithelial cells. Maturation of the matrix has been observed taking anywhere from 4-12 hours post secretion in *Ae. aegypti* (Perrone and Spielman, 1988). Successful entry into the epithelial cells proceeds with viral RNA replication at the endoplasmic

reticulum membrane. While the location of viral maturation can differ depending on the virus or mosquito species, it is generally accepted that newly propagated virions must be able to leave the midgut epithelium to reach the salivary glands later. SLEV virions were observed to accumulate within the midgut epithelium due to an intact basal lamina suggesting it acts as a barrier secondary barrier (Whitfield *et al.*, 1973). Weaver *et al.* (1988) observed that when the midgut of *Culiseta melanura* (Coquillett) was infected with eastern equine encephalitis virus (EEEV), detrimental changes to the basal lamina occurred. Weaver et al. (1988) speculated it was a mechanism employed by the virus to escape the midgut. It is assumed that the virus propagates again finding suitable tissue to infect to increase the chances of infecting the salivary glands.

1.7.2 Salivary Gland Barriers

To date there exists no molecular mechanism that explains both salivary infection and escape barriers sufficiently. Rosomer *et al*. (2005) postulated that the basal lamina of the salivary glands may resemble midgut epithelial basal lamina, acting as a physical barrier for virion entry and/or escape. Innate differences between lobes have been proposed as a potential reason for salivary gland infection. Juhn *et al*. (2011) showed that certain transcripts in *Ae. aegypti* glands localized only to the median or lateral lobes while others were found throughout and may play a role in determining susceptibility. Structurally, only the lateral lobes contain cuticular filamentous membrane extensions which have been found to be required attachment sites for Sindbis virus (SINV) (Ciano *et al*., 2014). The induction of cytopathic effect and salivary gland cell apoptosis by SINV has been thought to be a requirement for transmission of other viruses.

1.8 Mosquito Taxonomy and Biology

Mosquitoes are categorized in the family Culicidae, under the Order Diptera. These "True flies" are known to have a pair of wings and halteres. In Ontario, Canada alone, there are 69 species of mosquitoes with many acting as important or potential vectors for disease (Giordano *et al*., 2015). Known for their voracious appetite for blood, mosquitoes have long plagued humans for thousands of years. Their unique life cycle and wide preference of animals to feed from has led to the establishment of successful mosquito populations all over the world.

1.8.1 Life Cycle

Mosquitoes develop through 4 life stages: egg, larva, pupa and adult. The first 3 stages are aquatic while the adult stage is terrestrial. Generally, development from egg to newly emerged adult lasts 1 to 2 weeks. The rate of development and size of the adult are dependent on the amount of nutrients acquired by the larva and the temperature of the water they were reared in. Adult mosquitoes in the wild are assumed to live 1-2 weeks while those bred in captivity have been noted to live up to a month (Clements, 1993)

1.8.2 Egg Stage

Adult female mosquitoes with a mature batch of eggs generally seek damp environments to oviposit. Depending on the genus of the mosquito, the structure and placement of eggs can differ. *Aedes* mosquitoes lay their eggs individually on moist substrates near but above water level waiting for submersion (Gillett, 1956). *Culex* mosquitoes lay their eggs on the water's surface in a floating mass called a raft (Christopher, 1945). While both require approximately 2-3

days to hatch, *Aedes* eggs have evolved to withstand months of drought while *Culex* eggs lack this adaptation (Clements, 1993)

1.8.3 Larval Stage

Newly *hatched* Aedes and *Culex* larvae scrape or filter feed on organic substrates. Although aquatic, mosquito larvae fulfill their oxygen requirements by drawing air from the surface through a structure near their tail end known as a siphon. Larvae submerged to either eat or hide from predators, spending most of their time near the surface. During this stage, the larvae continually eat, grow and shed its exoskeleton 4 times, developing through sub stages known as instars. The 4th instar molts for the last time into the next developmental stage known as the pupa.

1.8.4 Pupal Stage

Pupae lose function of their mouthparts due to the fusion of the cephalothorax and relinquish their ability to feed but acquire a hardened cuticle to protect the developing adult mosquito within. Two trumpet like structures found dorsally replace the ventral siphon as the sole breathing apparatus. Although rigid in appearance, pupae still exhibit larvae-like mobility, being capable of submerging from the surface and escaping danger. When development has ceased, the pupa floats to the top and the adult mosquito emerges from the cuticle (Clements, 1993).

1.8.5 Adult Stage

Adult mosquitoes readily adapt to their terrestrial surroundings aided by their ability to fly. Males generally develop faster than females and are usually the first to emerge. Compared to the female, males tend to be smaller, have more plumose antennae, and can have elongated palpi depending on the genus. Both sexes have somewhat similar mouthparts and require energy from carbohydrate sources such as fruit or honeydew, however the distinguishing characteristic of blood feeding is female only. Mated female *Aedes* mosquitoes seek blood to mature their batch of eggs. Proteins and other factors from blood help to develop their eggs however, blood feeding in mosquitoes is not always required. Some species of *Culex* mosquitoes have autogenous females, producing eggs without the need for blood, unlike the anautogenous *Aedes* mosquito (Roubaud, 1929)

1.8.6 Blood Feeding

While not exclusive to mosquitoes, hematophagy or blood feeding is one of their most commonly associated characteristics. Using a variety of visual and sensory cues including CO_2, heat, and host-derived odors, hungry females locate potential blood meals from attractive animal hosts. After landing on a host the female readies her specialized mouth parts, the proboscis, revealing the stylets and shallowly penetrates the skin trying to locate suitable blood vessels. This process is also known as probing. When a suitable site has been located, the mosquito starts to feed by drawing blood. During probing and feeding, the mosquito salivates, releasing a variety or enzymes into the host to facilitate blood feeding. Drawn blood is immediately diverted to the insect midgut, where it is later processed and digested (Clements, 1993).

1.8.7 Salivary Glands Structure

Both male and female mosquitoes contain a pair of salivary glands located within the thorax, above the forelegs. In most species, each gland is tri-lobed which all converge to a single shared duct. A salivary gland consists of a short medial lobe flanked on both sides by two longer lateral lobes. The medial lobe consists of both a neck and distal region while the lateral lobes can be further divided into the proximal, intermediate, and distal regions. Each lobe is composed of a layer of epithelial cells that surround a primary duct (James and Rossignol, 1991). The pair are connected internally to the base of the proboscis via the salivary valve and thus saliva can readily be secreted during feeding.

1.8.8 Salivary Components

While salivary gland extracts from various insect species have been shown to contain approximately 120 different proteins only a small fraction of them have been properly characterized for their biochemical function (Almeras, 2010). The proteins that have been identified include the members of the 5'- nucleosidase, mucin and D7 proteins. The 5'-nucleosidase protein family consists of enzymes that inhibit the hemostatic response in vertebrate blood meals (Champagne, 1995). A well-known protein found in blood feeding species of mosquito saliva is the enzyme apyrase. This approximately 64 kDa sized protein (Francischetti, 2002) inhibits platelet aggregation by catalyzing the degradation of adenosine triphosphate (ATP) to adenosine monophosphate (AMP), limiting the availability of adenosine diphosphate (ADP), a stimulus needed for the hemostatic event to occur (Ribeiro, 1985). Mucins are glycoproteins found in mosquito saliva. These proteins are thought to lubricate the salivary canal as well as play a role in the modulation of an immune response in vertebrate hosts (Valenzuela,

2003). D7 proteins are unique as they are only found in the salivary glands of sand flies and mosquitoes (Valenzuela, 2002). There exist two types of D7 proteins: long D7 (28-30 kDa) found in sandflies and mosquitoes, and short D7 (15-20 kDa) found only in mosquitoes (Arca, 1999). These proteins play a role in inhibiting the activation of host plasma (Valenzuela, 2003). The protein hamadarin (16 kDa) had been shown to inhibit the plasma contact system, thus delaying the release of bradykinin, a peptide that mediates an inflammatory response (Isawa, 2002).

Chapter 2

Differential Potentiation of Flavivirus Infection Using Saliva Collected from a Variety of Female Mosquitoes

2.1 Abstract

As female mosquitoes feed on hosts for blood, they secrete saliva. This saliva contains a variety of proteins and factors that helps facilitate feeding. If infected with WNV, the virus is transmitted along with the saliva. Mosquito saliva potentiates *Flavivirus* infection in both *in vitro* and *in vivo* models; however, it remains unknown whether saliva from different species differentially potentiates infection. By inoculating the saliva of different mosquito species plus WNV onto Vero cells, plaque assays were used to study if saliva could differentially potentiate WNV infection. It was found that while there was no significant difference between *Ae. aegypti* and *Ae albopictus* saliva ($p=0.19$), more interestingly was that both saliva treatments had a significant reduction in plaques formed compared to virus alone ($p= 0.01$ and $p=0.00$). The presence of mosquito saliva appears to exert a protective effect *in vitro* when WNV is present.

2.2 Introduction

Whether it be drinking honeydew or imbibing a blood meal, female mosquitoes secrete saliva during feeding (Eliason, 1963; Ribeiro., *et al*., 1984). As they salivate, they release a variety of proteins and factors that exhibit a variety of functions. During blood feeding salivary components with anticlotting, antiplatelet and vasodilatory properties are secreted to facilitate the process (Ribeiro., *et al*., 1984). If the mosquito has a disseminated infection of a flavivirus, then potentially the virus can be secreted along with the saliva. Interestingly, mosquito saliva not only serves to help mosquitoes, but also seems to influence the virulence of flaviviruses in bitten hosts. Mice and chicken previously inoculated with mosquito saliva and then virus or given virus mixed with saliva showed increased viremia and had higher rates of mortality compared to those without saliva (Schneider *et al*., 2006; Schneider *et al*., 2007; Styer, *et al*., 2006; Styer *et al*.,

2011). This potentiation effect has been linked to changes in the immune response induced by factors found within the saliva (Schneider *et al*., 2010). It has been shown that mosquito saliva potentiates flavivirus infection in both *in vitro* and *in vivo* models (Conway *et al*., 2014), however it remains unknown as to whether saliva from different species differentially potentiates infection.

The present study attempts to determine whether saliva from different mosquitoes can differentially potentiate infection. SGE extracted from different mosquito species and mixed with WNV was used to inoculate Vero E6 Cells. Different concentrations of SGE was first used to determine whether it affected the timeline of cytopathic effect. This was then followed by comparing SGE from different species and whether it potentiated infection determined through plaque assay.

Understanding whether saliva from different species differentially potentiates infection allows researchers to focus vaccine development efforts. Traditionally, flavivirus vaccine development has targeted viral antigens, however recent vaccines designed to target arthropod salivary gland components have shown potential (Titus *et al*., 2006). By identifying species specific salivary factors that are implicated as primary contributors in potentiating infection, better multi- subunit vaccines can be made.

2.3 Methods

2.3.1 Mosquitoes

Culex pipiens f. *molestus, Aedes aegypti,* and *Aedes albopictus* colony mosquito eggs were obtained from Rutgers University. Eggs were hatched and the larvae reared in tray filled with dechlorinated tap water at 28°C ± 1°C with a photoperiod of 16:8 hours light dark cycle. Larvae were maintained on a larval diet consisting of ground Tetramin™ fish food and brewer's yeast, with the water being changed daily (Appendix). Pupae were then sorted and placed into emergence tanks. Newly emerged adult mosquitoes were then separated and placed in cages at 28°C ± 1°C with a photoperiod of 16:8 hours light dark cycle at 75% ± 5 % humidity. Adults were maintained on 10% sucrose (Appendix) soaked cotton pads changed daily.

2.3.2 Salivary Gland Dissection

Approximately 3-5 day old mosquitoes were used for salivary gland dissection. Salivary gland dissections were performed on a glass microscope slide place atop a chill table (BioQuip, United States) and viewed under a dissecting microscope (Leica, Germany). A drop of phosphate buffered saline (PBS) (Agbulos *et al*., 2016) was placed between the head and thorax to prevent desiccation during dissection. The head was gently pulled away from the body while pressure was applied to the thorax, revealing a pair of salivary glands. The glands were gently teased away from the head using fine probes and transferred in to a micro centrifuge tube filled with PBS on ice. Stock solutions of salivary gland extract (SGE) were made to a ratio of 1 salivary gland pair per 10 ul PBS. The sample solution was quickly freeze/ thawed 3 times, then centrifuged at 15,000 g for 5 minutes. The contents were then transferred to a 1.2 ml Corning

cryogenic vial, quickly frozen in liquid nitrogen and stored at -80˚C until needed. Human saliva was processed similarly to mosquito SGE.

2.3.3 Virus

The WN-NY99 was used to infect Vero E6 cells. WN-NY99 strain was obtained from The National Microbiology Laboratory (Winnipeg, MB, CAN) on behalf of the Public Health Agency of Canada. Stock titre of ZIKV used was 10^6 PFU. WN-NY99 was derived from a dead Chilean flamingo at the Bronx Zoo, New York, United States (Lanciotti *et al*., 1999). It was later isolated and passaged (p2) in Vero E6 cells. The stock titre of WNV used was 10^6 PFU. All virus concentrations were determined through plaque assay.

2.3.4 Cytopathic Effect Assay

To help initially determine whether differences in salivary concentrations contributed to cytopathic effects, Vero E6 cells were plated onto 6-well plates (Sarstedt, Germany) and maintained with culture media (Dulbecco's Modified Eagle Medium (DMEM) High Glucose high glucose supplemented with 10% Fetal bovine serum (FBS) and 1% Penicillin Streptomycin L-Glutamine (PSG), which was adapted from Eagle, 1995 (Agbulos *et al*., 2016). Cells were inoculated with mosquito SGE and virus once confluency reach >90%. Prior to inoculation, media within each well was removed, and cells were gently washed with warmed (37°C) PBS. Ten-fold serial dilutions of SGE were prepared up to a dilution of factor (d.f.) of 100. Inoculations were carried out as described below:

Well 1 (top left): 90 µL of stock SGE and 10 µL of stock WNV. Well 2 (top middle): 90 µL of dilution factor (d.f.) 10 SGE and 10 µL of stock WNV. Well 3 (top right): 90 µL of d.f 100 SGE

and 10 µL stock WNV. Well 4 (bottom left): 90 µL of stock human saliva and 10 µL of stock WNV. Well 5 (bottom middle): 100 µL of sterile PBS. Well 6 (bottom right): 90 µL of sterile PBS and 10 µL of stock WNV.Cells were left with the inoculum for 30 minutes at 37°C, with the plates being rocked every 10 minutes. After 30 minutes, the inoculum was removed from each well and 2 mL of culture media was added. Cells were observed for cytopathic effect for 5 days.

2.3.5 SGE Treatment Plaque Assay

Vero E6 cells were plated onto 6-well plates (Sarstedt, Germany) and maintained with culture media (DMEM high glucose supplemented with 10% FBS and 1% PSG). Cells were inoculated with mosquito SGE and virus once confluency reach >90%. Prior to inoculation, media within each well was removed, and cells were washed gently with warmed (37°C) PBS. SGE in a 1:1 ratio with PBS was used. Inoculations were carried out as described below: Wells 1 (top left) and 4 (bottom left) were each inoculated with 499 µL of 10^6 PFU WNV and 1 uL of *Ae. aegypti* SGE. Wells 2 (top middle) and 5 (bottom middle) were inoculated with 499 uL 10^6 PFU WNV and 1 uL of *Ae. albopictus* SGE. Well 3 (top right) was inoculated with 500 uL of prepared media acting as the negative control Well 6 (bottom right) was inoculated with 499 uL of 10^6 PFU WNV and 1 uL of PBS acting as the positive control.

Plates were incubated for 1 hour at 37°C, 5% CO^2, being shaken every 15 minutes (n=20). After 1 hour, the inoculum was removed, washed with PBS, and covered with 2 mL of a 1:1 2% DMEM and 3.4% Carboxymethyl cellulose (CMC) warmed, overlay solution (Agbulos *et al*., 2016). The plate was incubated at 37°C, 5% CO^2 for 4 days. The overlay was removed and the cells were gently washed with warmed PBS. Each well received 1 mL of crystal violet staining solution and were plates were left for 30 minutes (Agbulos *et al*., 2016). Staining

solution was then removed and the wells were rinsed with warm water to reveal plaques which were counted after drying.

2.3.6 Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A modified SDS- PAGE was performed based on the method of Laemmli (1970) using 12% Mini PROTEAN TGX Precast Gels (Bio- Rad). To prepare samples for loading, 13 µl of salivary gland extract was mixed with 27 µl of SDS – PAGE sample buffer (Appendix). Samples were initially heated to 95˚C using a Mastercycler Thermocycler (Eppendorf, United States), then cooled to room temperature prior to loading. PageRuler Prestained Protein Ladder (Thermo Fisher Scientific, United States) was loaded alongside the samples. Approximately 10 µl of ladder and 26 µl of sample were loaded into the gel wells. The gels were electrophoresed at 200 V continuously until the dye front and ladder reached the end of the gel. After electrophoresis, the gels were then stained using ProtoBlue Safe Colloidal Coomassie G-250 stain following the manufacturer's instruction (National Diagnostics, England) Newly stained gels were photographed and visualized using the Gel Doc EZ Imager (Bio-Rad, United States). Protein bands were analyzed and annotated using the software Image Lab to determine the molecular weight of each band in kDa (Bio-Rad).

2.4 Results

SGE from *Cx. pipiens molestus*, *Ae. aegypti*, and *Ae. albopictus* was isolated from dissected mosquito salivary glands and the proteins visualized using SDS PAGE gel electrophoresis. Prominent protein bands with similar sizes were observed between *Ae. aegypti* and *Ae. albopictus*, with dark, thick paired bands being located at the ~150 kDa, ~40 kDa and ~

22 kDa range (Figure 1A, B, and C). *Cx. pipiens molestus* bands were not as prominent, with banding patterns (Figure 1C) not matching across all three species compared to both *Aedes* lanes. Numerous faint bands with various weights were noted across all three species.

In the cytopathic effect assays, only *Cx. pipiens molestus* SGE was used. Generally, different concentrations of SGE did not appear to accelerate cytopathic effect before 5 days when cells were inoculated with both virus and SGE (Figure 2). Originally out of 12 plates only 4 had Well 1 appeared more pink compared to Wells 2 and 3 and 6 (Figure 2). The assay was repeated with 8 new plates, where the Well 1 discrepancy could not be repeated and observed.

In the SGE Treatment plaque assays, both *Ae. aegypti* and *Ae. albopictus* SGE (1 paired gland per well) were used. After 4 days, wells treated with virus alone showed significantly more plaques (22.4 ± 0.9) than either *Ae. aegypti* (17.2 ± 1.4) or *Ae. albopictus* (14.8 ± 1.1) SGE treatments (Figure 3). Virus alone compared to *Ae. aegypti* SGE with virus treatment was significantly different (**two sample t**(23)=2.7, **p**= 0.01). Virus alone compared to *Ae. albopictus* SGE with virus was significantly different (**two sample t**(19)=4.5, **p**= 0.00). *Ae. aegypti* SGE with virus treatment compared *Ae. albopictus* SGE with virus treatments was not significantly different (**two sample t**(32)=1.3, **p**= 0.19).

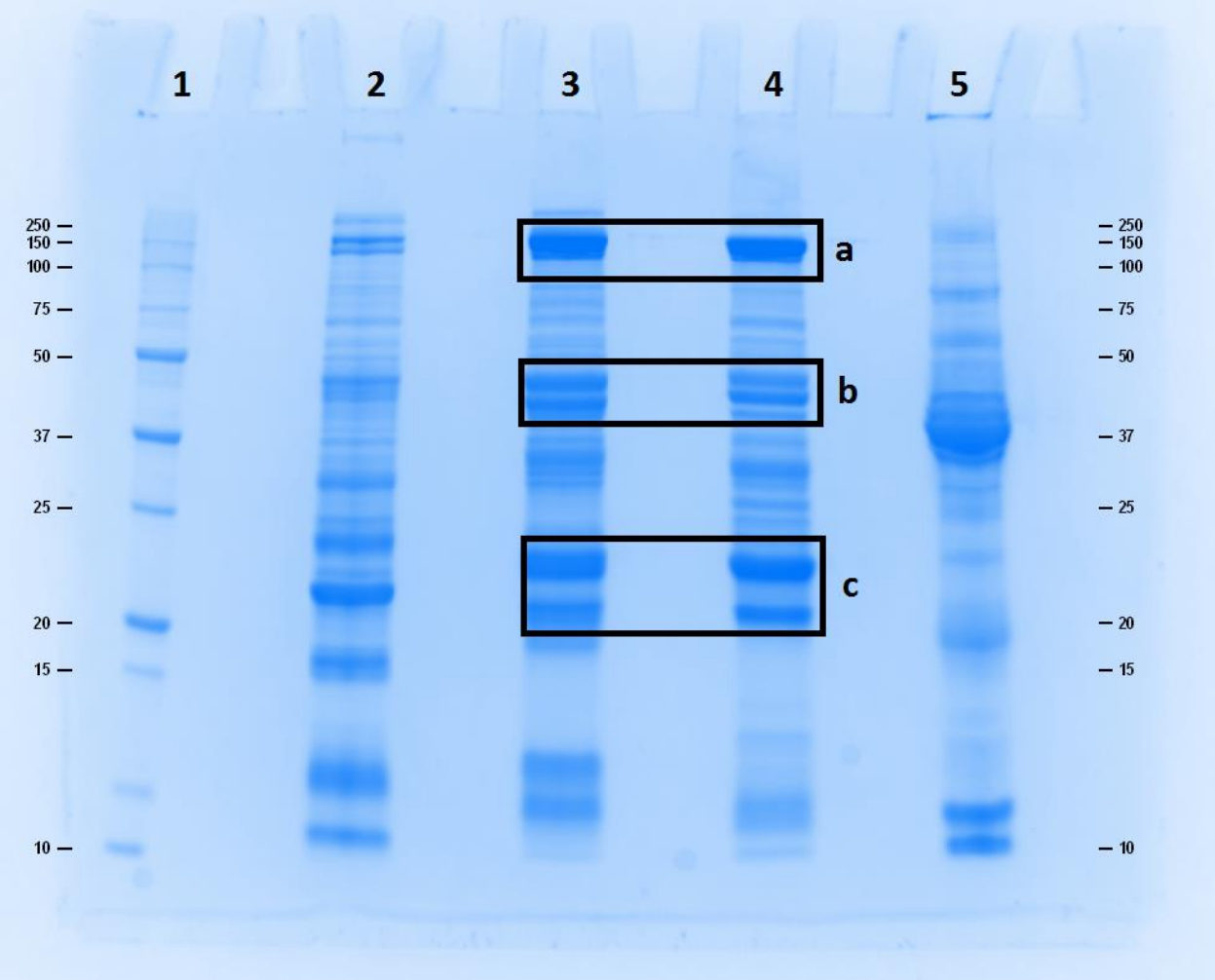

Figure 1. SDS PAGE Gel Electrophoresis of Mosquito SGE in a 12% polyacrylamide gel. Lane 1: PageRuler Unstained Protein Ladder (Thermo Scientific), Lane 2: *Cx. pipiens molestus* SGE. Lane 3: *Ae.aegypti* SGE. Lane 4: *Ae. albopictus* SGE. Lane 5: Human saliva. Lanes 3 and 4 observed to be very similar compared to Lane 2. Common prominent bands located around 150 (a), 40 (b) and 22 (c) kDa range. Multiple smaller bands not shared among the 3 mosquito lanes were also observed.

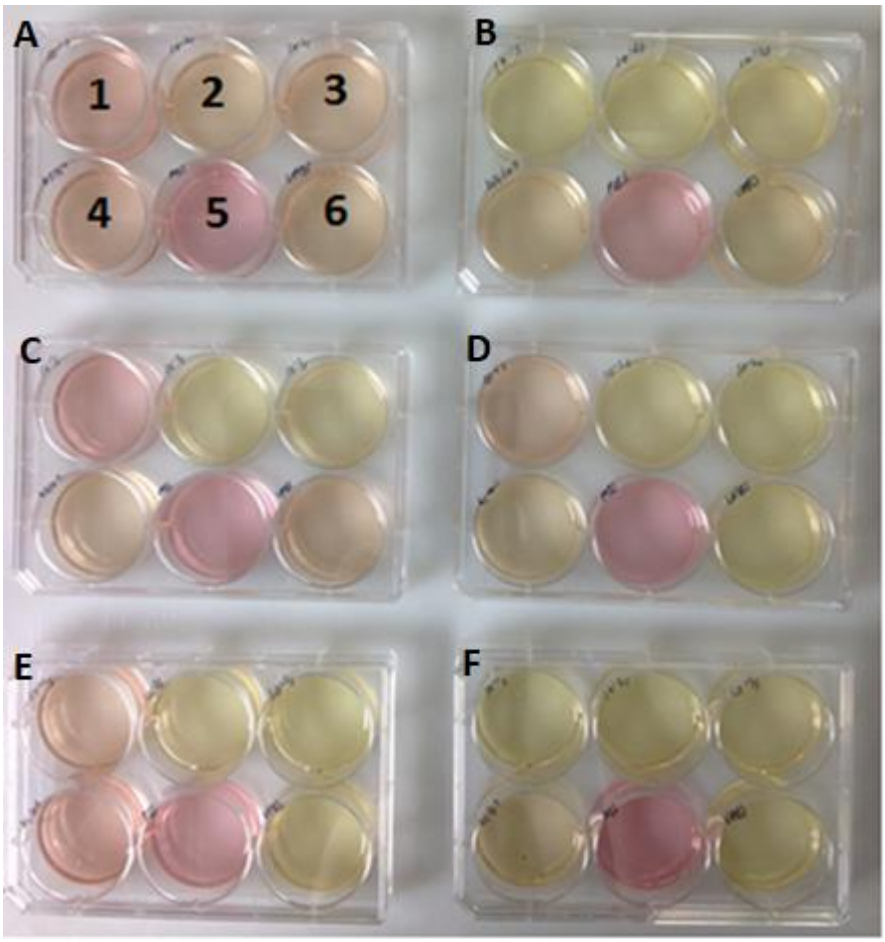

Figure 2. Vero Cells Treated with Serial Dilutions of *Cx. pipiens molestus* SGE and WNV. Well 1: 90 µL of stock SGE and 10 µL of stock WNV. Well 2: 90 µL of dilution factor (d.f.) 10 SGE and 10 µL of stock WNV. Well 3: 90 µL of d.f 100 SGE and 10 µL stock WNV. Well 4: 90 µL of stock human saliva and 10 µL of stock WNV. Well 5: 100 µL of sterile PBS. Well 6: 90 µL of sterile PBS and 10 µL of stock WNV. Portion of plates (n=20) represented after 5 days post infection (d.p.i.). Cytopathic effect visualized by yellow colour. It was observed that Well 1 for some plates (Figure 2 A, C, D, and E) showed less cytopathic effect then Wells 2, 3 and 6 (Figure 2 B and F).

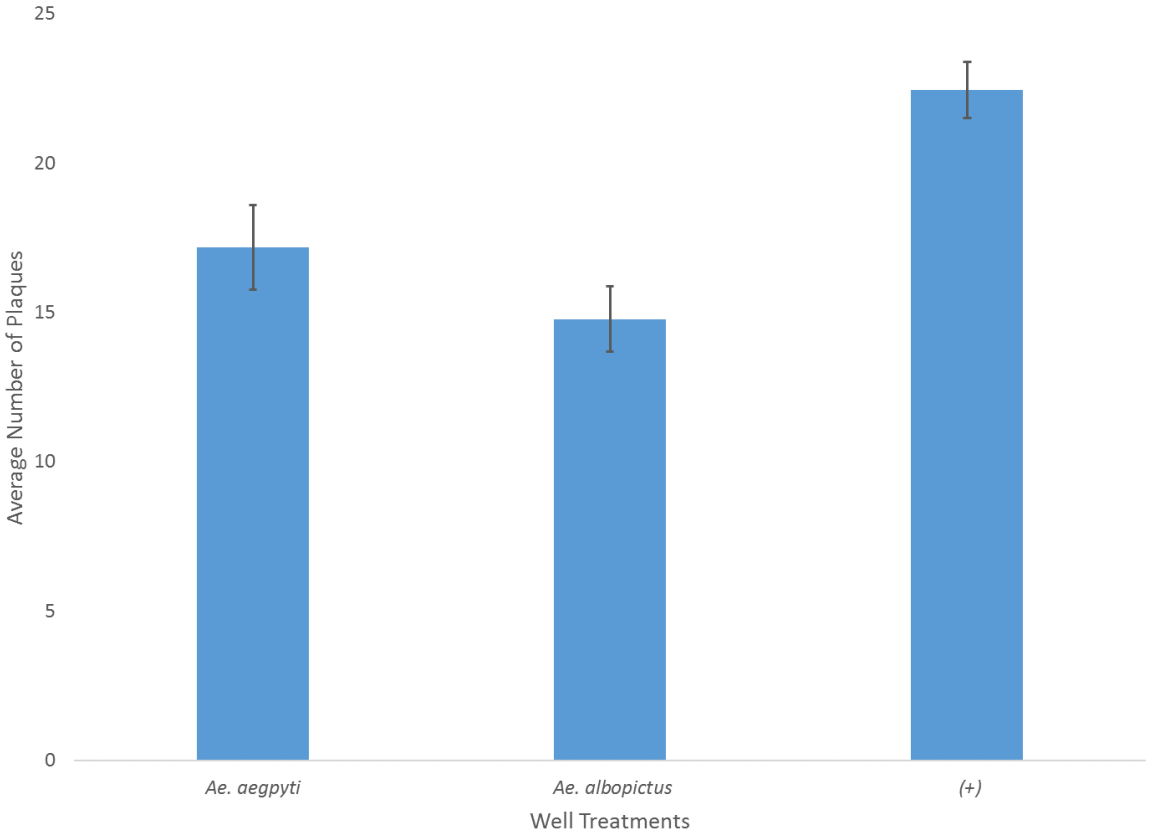

Figure 3. Vero Cells Treated with either *Ae. aegypti* or *Ae. albopictus* SGE and WNV. SGE treatment from either species reduced the number of plaques formed when inoculated with WNV compared to virus alone. A Two-Tailed T-Test assuming unequal variance was used to determine significance. The average number of plaques found in wells inoculated with both *Ae. aegypti* SGE and WNV (n=18) was 17.2 plaques per well. The average number of plaques found in wells inoculated with both *Ae. albopictus* SGE and WNV (n=18) was 14.8 plaques per well. The average number of plaques found in wells incubated with WNV alone (n=9) was 22.4 plaques per well. *Ae. aegypti* wells appeared to have more plaques compared *Ae. albopictus* wells (p=0.19). Virus alone wells had significantly more plaques than both the *Ae. aegypti* (p=0.01) and *Ae. albopictus* (p=0.00) wells.

2.5 Discussion

Our goal was to determine whether there was a differential potentiation of WNV in vitro using SGE from different mosquito species. Differences in observed proteins found in SGE between different species was expected, as *Cx. pipiens molestus* was less related than both *Aedes* species used, thus having a different profile of SGE proteins (Figure 1). This is most likely due to salivary proteins being more related between species in the same genus rather then across genera. Slight differences in weights can be attributed to differences in protein processing such as glycosylation patterns.When comparing the gel results to literature, there are differences in bands observed, specifically number, intensity, and location of. This can be attributed to differences in protocols such as salivary gland dissection/ preparation, SDS PAGE running conditions, or to a lesser extent innate differences in the sialome of different lab colonies used (Almeras *et al.*, 2010; Arca *et al.*, 2007).

Our results suggest that mosquito saliva in various concentrations did not appear to alter when cytopathic effect *in vitro* occurred after (Figure 2). Discrepancies in 4 plates (n=12) showed that the highest concentration of *Cx. pipiens molestus* SGE mixed with virus resembled negative control more so than the positive control, appearing to exert a protective effect. A new experimental replicate (n=8) could not repeat the previous results and thus the discrepancy could not be explained.

Our results suggest SGE from uninfected *Aedes* mosquitoes seem to reduce the number of plaques formed in plaque assay when mixed with WNV when compared to WNV alone. We showed that both *Ae. aegypti or Ae. albopictus* SGE mixed with WNV resulted in fewer plaques (17.2 and 14.8 plaques respectively), compared to the WNV treatment alone (24.4 plaques) (Figure 3). No significant difference was found when comparing the effect of *Ae. aegypti* versus

Ae. albopictus SGE (p=0.19), however both SGE treatments were significantly different compared to control (p= 0.01 and p=0.0002) (Figure 3). While minor differences in plaques can be attributed to differences in SGE between mosquito species, the protective effect differs from literature.

Numerous studies have found that both *in vivo* and *in vitro*, mosquito saliva did positively potentiate infection. *In vivo* studies using a variety of animals and different viruses noted increases in viremia and mortality (Cox *et al*., 2012; Schmid *et al*., 2016; Schneider and Higgs, 2008; Schneider *et al*., 2007; Styer *et al*., 2011). Conway *et al*. (2014) found that the serine protease activity of SGE mediates infection in vitro by increasing surface attachment of DENV to mouse embryonic fibroblast cells (MEF) by proteolyzing the cellular matrix. Few studies have shown the potential protective effect of insect saliva. Reisen *et al*. (2000) showed that chickens infected with SLEV via syringe inoculation responded with a higher immune response, detected with enzyme immunoassay and plaque reduction neutralization tests, compared to infective mosquito bite. *In vivo* models using Malaria and Leishmaniasis parasites (Donovan *et al*., 2007; Kamhawi *et al*., 2000) showed that the protective effect mosquito saliva seems to induce is linked host immunomodulatory changes (Donovan *et al*., 2007). The protective effects of mosquito saliva may have been selected over time as a mechanism for Flaviviruses to infect but not kill hosts, increasing its fitness.

A component of SGE from uninfected mosquitoes may play a role in reducing the virulence of WNV infection in vitro. Mosquitoes that naturally acquire viral infections mediate an immune response that can lead to the upregulated expression of antimicrobial peptides (Xi *et al*., 2008). These defenses are maintained at a basal level even when no infection is present. Girard *et al*. (2010) showed that *Cx. quinquefasciatus* infected with WNV had an increase in

transcript levels associated with immunity, transcription and cellular detoxification and decreases associated with salivary gland proteins and D7 protein family members. Xi *et al*. (2008), also noted a reduction in the AMP SRPN10A (serpin) in infected mosquito midguts. Mixing WNV and SGE from uninfected mosquitoes may alter potentiation due to levels of AMP SRPN10A present, thus hindering the virulence of WNV. Differences in when SGE is added may play a role in affecting potentiation. Some studies inoculate SGE first and then add the virus after a waiting period (Conway *et al*., 2014). Perhaps mixing SGE with virus initially makes WNV less likely to attach to mammalian cells in vitro. Using DENV and SGE from both *Ae. aegypti* and *Ae. polynesiensis* (Marks), Cao-Lormeau (2009) found that several SGE proteins could bind to DENV. WNV mixed with SGE may be inhibited from future receptor attachment due to being competitively bound with pre-existing SGE proteins.

Although some mosquito salivary proteins have been characterized since their discovery, hundreds remain unstudied. These unknown proteins may be involved in the potentiation of viral infection as well as exerting protective effects. Future research into differences in interspecies shared salivary gland proteins as well as unique proteins will help elucidate their role in potentiating viral infection both *in vivo* and *in vitro*. Furthermore, comparative studies using saliva from a variety of mosquitoes as well as different viruses should be implemented to better understand specific protein roles. Multi-subunit vaccine research focusing on proteins that may be responsible for inducing protective effects is counterproductive.

Chapter 3

Zika Virus: Quantification, Propagation, Detection, and Storage

3.1 Author Contributions

Personal contributions to the following published work includes: development and troubleshooting of Basic Protocol 1- Quantification of Zika Virus by Plaque Assay and Basic Protocol 2-Generation and Purification of Zika Virus Stocks, photographing plaque assay results for publication, written Introduction, minor additions to the Commentary section, and manuscript editing.

Zika Virus: Quantification, Propagation, Detection, and Storage

Darrell S. Agbulos,[1,4] Larissa Barelli,[1,2,4] Bryan V. Giordano,[1,4] and Fiona F. Hunter[1,2,3,4]

[1]Centre for Biotechnology, Brock University, St. Catharines, Ontario, Canada
[2]Entomogen Incorporated, St. Catharines, Ontario, Canada
[3]Department of Biological Sciences, Brock University, St. Catharines, Ontario, Canada
[4]These authors contributed equally to this work

Zika virus (ZIKV), belonging to the family Flaviviridae, genus *Flavivirus*, is an arthropod-borne virus that was first discovered from the Zika forest in Uganda in 1947. Recent outbreaks in South America have linked ZIKV to cases of microcephaly and Guillain-Barré syndrome in humans. With the increased interest in ZIKV, protocols must be established to facilitate proper research. Here we describe the laboratory techniques required to quantify, propagate, and store ZIVK. We also review the proper safety protocol for the handling of ZIKV, which is classified as a Biosafety Level 2 pathogen by the United States Centers for Disease Control and Prevention.

Keywords: Zika virus • infection • plaque assay • detection • Vero cells

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INTRODUCTION

Zika virus (ZIKV; family Flaviviridae, genus *Flavivirus*) is an enveloped, positive-sense, single-stranded RNA virus. Its genome (~11 kb) encodes three structural proteins (one capsid [C] and two envelope proteins [M, E]), as well as seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5; Kuno and Chang, 2007). ZIKV was first isolated in 1947 from a sentinel rhesus monkey from the Zika forest in Uganda (Smithburn, 1952). It was subsequently detected in *Aedes africanus* mosquito pools in 1947 and then in humans in 1952 (Dick, 1952; Dick et al., 1952). While the reservoir host has yet to be determined (WHO, 2016), it is assumed to be propagated in nature through an enzootic cycle involving mammalian hosts, such as primates, and insect vectors, primarily mosquitoes of the *Aedes* genus (Marchette et al., 1969; Fagbami, 1979; McCrae and Kirya, 1982; Akoua-Koffi et al., 2001). Originally endemic to Africa, ZIKV spread throughout parts of Asia eventually being found in Southeast Asian human populations and mosquito pools. Recent outbreaks in Yap Island, Micronesia and the French Polynesian islands prompted investigation due to the similarity in symptoms to other related mosquito-borne flaviviruses such as dengue, West Nile, and yellow fever viruses (Duffy et al., 2009). Renewed interest in ZIKV has once again emerged alongside the 2015 Brazilian outbreak due to the potential link between the virus and its potential teratogenic and neurodegenerative effects.

This unit describes the methods required for the quantification (Basic Protocol 1), propagation, and storage (Basic Protocol 2) of ZIKV stock solutions. Procedures

for maintenance of the required cell lines are also described (Support Protocols 1 and 2).

CAUTION: ZIKV is a Biosafety Level 2 (BL-2) pathogen, with the exception of the United Kingdom which has classified ZIKV as a Biosafety Level 3 (BL-3) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. Potential aerosol generating procedures should always be performed in a certified biological safety cabinet (BSC). See *UNIT 1A.1* and *APPENDIX 1B* for more information.

CAUTION: Sexual transmission of ZIKV has been reported (Foy et al., 2011; Musso et al., 2015), and ZIKV virions have been detected in semen samples of infected men (Atkinson et al., 2016; Mansuy et al., 2016). All workers and their sexual partners should be made aware of the risks prior to working with ZIKV. Given that the potential teratogenic effects of ZIKV remain to be elucidated, additional safety precautions should be considered for pregnant workers, workers whose partners are pregnant, or workers trying to start a family. All workers should consult the updated guidelines set out by the Centers for Disease Control and Prevention (Oster et al., 2016).

NOTE: We recommend workers wear a second layer of gloves when working with ZIKV. The second pair is to be removed prior to exiting the BSC. Workers should take extra precaution with pipet tips or other sharp objects that are capable of puncturing the skin. A 1% Virkon solution can be used as a disinfectant in addition to 70% ethanol. Liquid waste containers can be equipped with a small funnel and 1 to 2 in. of 1% Virkon to minimize splashing and production of aerosols.

BASIC PROTOCOL 1

QUANTIFICATION OF ZIKA VIRUS BY PLAQUE ASSAY

Plaque assay is the gold standard for quantification of viral stock solutions and virus-containing samples. The assay described here is applicable to determining the titer of ZIKV stocks or the supernatant of infected cells and animal tissue homogenates. This protocol utilizes carboxymethylcellulose sodium salt (CMC) to overlay the infected cell monolayers. CMC should be prepared 1 to 2 days prior to experimentation.

Materials

Vero E6 cell line (ATCC #CRL-1586; see Support Protocol 1)
Vero E6 culture medium (see recipe)
Dulbecco's modified Eagle medium with 2% fetal bovine serum (DMEM/2% FBS; see recipe)
Dulbecco's phosphate-buffered saline (DPBS; see recipe)
CMC/DMEM overlay (see recipe)
Samples to be assayed
Crystal violet solution (see recipe)

6-well tissue culture plates
Cell culture incubator set to 37°C and 5% CO_2
37°C water bath
Camera, to photograph 6-well plates

Seeding well plates and sample preparation

1. Seed Vero E6 cells in 6-well tissue plates with 1.5 ml/well (1.5×10^5 cells/well). Incubate the plates in a cell culture incubator for 2 to 3 days or until the desired confluency has been reached.

   For best results use well plates that are 90% to 95% confluent (see Critical Parameters and Troubleshooting).

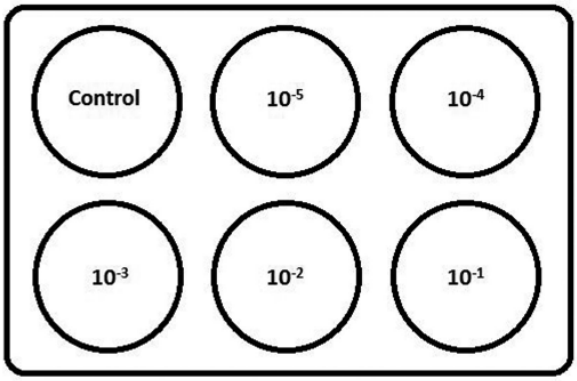

Figure 15D.4.1 Plaque assay 6-well plate setup.

2. Set the water bath to 37°C and warm DMEM/2% FBS, DPBS, and CMC/DMEM overlay. Thaw samples to be assayed in a BSC.
3. Prepare 5 serial dilutions of each sample by combining 200 µl sample and 1.8 ml DMEM/2% FBS. Mix each dilution thoroughly by pipetting up and down for a few seconds. Use a new pipet tip between each dilution.

   This will make 10^{-1} to 10^{-5} dilutions. You may need to further dilute the sample depending on the viral concentration (see Critical Parameters and Troubleshooting). There is enough final dilution to test the sample in triplicate.

4. Remove the 6-well plates from the incubator and place them inside the BSC.
5. Remove the Vero E6 culture media from each well, and add 1 to 2 ml DPBS to wash the cells.

   Do not apply DPBS with high pressure directly to the cells as this may cause the monolayer to disassociate from the plate. Gently add DPBS down the side of each well and rock the plates backward and forward and left to right for 1 to 3 min to aid in the removal of cellular debris and excess media.

6. Remove and discard the DPBS. Use a fresh pipet tip for each plate.

Infection of the monolayer

7. Add 500 µl DMEM/2% FBS to the top-left well of each plate as a control. Add 500 µl of each dilution to the side of a well working from left to right from highest dilution to lowest dilution.

   See Figure 15D.4.1 for best practice. This plate setup saves time and serological pipet tips when working from highest dilution factor to lowest. Work quickly to avoid the cells from drying out.

8. Incubate the infected well plates at 37°C and 5% CO_2 for 1 hr with gentle rocking every 15 min to spread the viral inoculum evenly across the monolayer.
9. After the 1 hr adsorption period, discard the virus suspension working from the lowest concentration to the highest. Use a fresh pipet tip for each well.

Addition of the overlay

10. Wash the wells with 1 to 2 ml DPBS as described in step 5.
11. Add 2 ml CMC/DMEM overlay to each well.

    Apply the overlay with low pressure down the side of the well plate.

12. Incubate the plates at 37°C and 5% CO_2 for 5 days (120 hr).

    Do not disturb the plates during this incubation period. Set the plates in the back corner of the incubator to avoid temperature fluctuations from repeatedly opening and closing the incubator during routine work.

13. After the 5 day incubation period, remove and discard the overlay in a BSC. Use a fresh pipet tip for each plate.

14. Remove excess overlay by washing the cells with 2 to 3 ml DPBS.

    Incubate the well plates at room temperature for 5 to 10 min with gentle rocking every 2 min, or until the overlay is fully dissolved. Well plates can also be placed in the incubator to aid in this process.

15. Remove and discard the wash solution.

Staining

16. Add 2 ml crystal violet solution to each well working from the lowest dilution to the highest. Use a fresh pipet tip for each well.

    Apply the crystal violet solution with low pressure down the side of the well plate.

17. Incubate the plates at room temperature for 30 min with gentle rocking every 10 min.

    Turn off the lights in the BSC or cover the plates with aluminum foil as crystal violet solution is light sensitive.

18. After incubation remove the staining solution and gently wash the wells with tap water from the sink.

19. Invert the plates and let dry on absorbent pads prior to plaque visualization.

    Let dry for 1 to 2 hr or overnight prior to counting plaques.

Estimation of viral titer

20. Select the dilution that has produced 30 to 100 plaques, and count the number of plaques for each replicate.

    See Critical Parameters and Troubleshooting if counts are >100 or the monolayer is not confluent.

21. For each sample calculate the average number of plaques for that dilution.

22. Calculate the plaque forming units per ml (PFU/ml) for each sample:

$$\text{PFU/ml} = \frac{\text{average number of plaques}}{(\text{dilution factor of well})(\text{volume of inoculum per plate})}$$

23. Take photos of the results (optional but recommended), and store the plates in the dark at room temperature.

GENERATION AND PURIFICATION OF ZIKA VIRUS STOCKS

BASIC PROTOCOL 2

ZIKV can be easily propagated in mammalian and mosquito cell lines (e.g., Vero E6 and C6/36, respectively). Here we describe propagation using the Vero E6 cell line; however, this protocol can be modified to suit the C6/36 cell line. Various strains of ZIKV can be purchased from the ATCC (*http://www.atcc.org/en.aspx*). The authors recommend quantifying any initial ZIKV stock solutions prior to experimentation or manipulation (Basic Protocol 1). Once the titer is known, proceed to propagation. A

confluent monolayer of cells is infected at a low multiplicity of infection (MOI) to reduce the number of defective virus particles. ZIKV stock solutions are brought to a final FBS concentration of 20% prior to long-term storage.

Materials

Vero E6 cell line (ATCC #CRL-1586; see Support Protocol 1)
Vero E6 culture medium (see recipe)
Optional: C6/36 cell line (ATCC #CRL-1660; see Support Protocol 2)
Optional: C6/C3 culture medium (see recipe)
DMEM/2% FBS (see recipe)
DPBS (see recipe)
Zika virus strain
Tris·Cl, NaCl, EDTA solution (TNE; see recipe)
TNE/25% glycerol (see recipe)

Cell culture incubator set to 37°C and 5% CO_2
37°C water bath
75-cm^2 tissue culture flasks (sizes vary with personal needs)
10-ml and 50-ml conical tubes
Centrifuge
2.0-ml cryotubes with O-ring
Optima XL-100 K ultracentrifuge with SW 28 swinging bucket rotor
5-ml and 25-ml serological pipets

Sample and monolayer preparation

1. Set the water bath to 37°C and warm DMEM/2% FBS, DPBS, and Vero E6 culture medium. Thaw ZIKV stock solution to be used for infection in the BSC.

   All solutions that come into contact with the cells should be previously warmed to 37°C.

2. Begin with eleven 75-cm^2 tissue culture flasks that are ~90% to 95% confluent (see Support Protocol 1).

   The authors recommend propagating ten flasks at a time. The eleventh flask is used for cell count determination.

3. Use one of the 75-cm^2 flasks to get a cell count.

4. Using an MOI of 0.01 and the calculated cell count, calculate the volume of viral inoculum required:

$$\text{MOI} = \frac{(\text{PFU/ml})(\text{volume of inoculum})}{\text{number of cells}}$$

   Select a previously titered ZIKV stock solution. Insert the PFU/ml of this stock solution into the equation above, and then solve for volume of inoculum.

5. Transfer the calculated volume of ZIKV stock solution from step 4 into a 10-ml conical tube. Bring the final volume of inoculum up to 5 ml with DMEM/2% FBS. Repeat to obtain ten volumes of inoculum.

6. Remove and discard growth media from the remaining 10 flasks, and rinse the flasks with 5 to 10 ml DPBS.

   Do not apply the DPBS with high pressure directly to the cells as this may cause the monolayer to disassociate from the culture flask. Gently add the DPBS down the neck of the flask and rock backward and forward and left to right for 1 to 3 min to aid in the removal of cellular debris and excess media.

7. Remove and discard the wash solution.

Infection of the monolayer

8. Add 5 ml of the previously prepared viral inoculum (step 5) to each of the 10 flasks.
9. Incubate the flask at 37°C and 5% CO_2 for 1 hr with gentle rocking every 15 min to spread the viral inoculum evenly across the monolayer.
10. After the 1 hr adsorption period, add 4 ml Vero E6 culture medium.
11. Incubate the flask at 37°C and 5% CO_2 for 40 to 48 hr.

    Do not disturb the flask during this incubation period. Set the flask in the back corner of the incubator to avoid temperature fluctuations from repeatedly opening and closing the incubator during routine work. Incubation should not continue once cytopathic effect is observed (see Critical Parameters and Troubleshooting).

Harvesting Zika virus

12. After the incubation period transfer the media to a 10-ml conical tube, and centrifuge 10 min at 1,300 × *g*, 4°C.
13. Remove and pool the supernatant; mix thoroughly. Set aside 200 µl for determination of viral titer by plaque titration assay (Basic Protocol 1).
14. Aliquot pooled supernatant in 1-ml increments into 2.0-ml cryotubes with an O-ring.
15. Store samples at −80°C until further use or proceed to virus purification if required.

    For long-term storage bring the remaining volume to a final concentration of 20% (v/v) FBS.

Virus purification

This protocol was adapted from *UNIT 15D.3* (Brien et al., 2013). Ensure you are familiar with the ultracentrifuge manufacturer's instructions prior to attempting this protocol.

16. Set the ultracentrifuge rotor temperature to 4°C.
17. Transfer 25 ml of the pooled virus supernatant prepared during step 14 to a 50-ml conical tube.
18. Add 5 ml TNE/25% glycerol to the bottom of each centrifuge tube.

    Add the TNE/25% glycerol as close to the bottom of the tube as possible to maximize the volume of virus suspension above it.

19. Fill the centrifuge tubes as close to the top of the tube as possible with DMEM/2% FBS, and balance centrifuge tubes to within 0.1 g.
20. Place the tubes into the ultracentrifuge.

    Disinfect the outside of the centrifuge tubes prior to transferring them into the centrifuge. Ensure the biosafety carriers are balanced.

21. Pellet virions through the glycerol cushion by centrifuging the samples 3 hr at 110,500 × *g*, 4°C.
22. After centrifugation remove and discard the top-most layer of supernatant using a sterile 25-ml serological pipet.

    Leave ~5 ml TNE/25% glycerol in the conical tube.

23. Remove 4 ml TNE/25% glycerol using a 5-ml serological pipet.
24. Resuspend virus pellet in 1 ml TNE.

The virus pellet will not be visible in the centrifuge tube. To aid resuspension use a 1-ml pipet tip to scrape the sides of the conical tube, moving in small circular motions.

25. Combine virus solutions and aliquot in 1-ml increments into 2.0-ml cryotubes with an O-ring.

26. Proceed to Basic Protocol 1, or store samples at −80°C until further use.

DETECTION OF ZIKA VIRUS BY REAL-TIME REVERSE TRANSCRIPTION PCR

BASIC PROTOCOL 3

Real-time reverse transcription polymerase chain reaction (qRT-PCR) is a quick and efficient method for detecting the presence of viral gene segments. This protocol describes the detection of ZIKV RNA levels by qRT-PCR using iTaq universal probe one-step master mix. Primer/probe pairs can be purchased from Sigma (*http://www.sigmaaldrich.com/life-science/custom-oligos.html*) and resuspended at a final concentration of 100 μM. Aliquots of 10 μM working primer/probe stocks should be stored at −20°C. Positive controls can be prepared from previously quantified ZIKV stock solutions of titers 10^5 PFU/ml or higher. Perform routine RNA isolation and serial dilutions to obtain a positive control stock solution. Perform serial dilutions to obtain working stocks that range from 10^{-1} to 10^{-3} dilutions. Store positive controls at −80°C. This protocol can be modified for a two-step master mix and to accommodate different fluorophores.

Materials

Primers and probes:
Based on Zika virus strain MR 766 (GenBank #AY632535; Lanciotti et al., 2008)
Zika 835 fwd: 5′-TTGGTCATGATACTGCTGATTGC-3′
Zika 911c rev: 5′-CCTTCCACAAAGTCCCTATTGC-3′
Zika 860 FAM probe: 5′-CGGCATACAGCATCAGGTGCATAGGAG-3′
Zika 1086 fwd: 5′-CCGCTGCCCAACACAAG-3′
Zika 1162c rev: 5′-CCACTAACGTTCTTTTGCAGACAT-3′
Zika 1107 FAM probe 5′-AGCCTACCTTGACAAGCAGTCAGACACTCAA-3′
iTaq Universal Probe One-Step Master Mix (e.g., Bio Rad, cat. no. 1725131)
iTaq-RT
Nuclease-free water

MicroAmp Fast Optical 96-Well Reaction Plate (e.g., ThermoFisher, cat. no. 4346907)
Vortex
Multichannel pipet
Optically transparent sealing film (e.g., ThermalSeal RT, Excel Scientific, cat. no. TSRT2100)
Real-time PCR detection system

1. Plan the layout of the 96-well plates, and calculate the appropriate volume of master mix required for the number of samples, including at least three positive controls and one negative control, using Table 15D.4.1.

   The authors recommend running 84 samples for each 96-well plate. This leaves room for three positive controls and one negative control in triplicate.

2. Thaw primers, probes, and RNA on ice.

3. Prepare master mix on ice.

4. Gently vortex the master mix for 30 to 60 sec, and add 20 μl to each well.

Table 15D.4.1 iTaq Universal Probe Master Mix Recipe

Reagents	1×	106×[a]
PCR master mix	12.5 µl	1325 µl
iTaq-RT	0.5 µl	53 µl
10 µM fwd	1.0 µl	106 µl
10 µM rev	1.0 µl	106 µl
10 µM probe	0.5 µl	53 µl
Nuclease free water	4.5 µl	477 µl
Total master mix volume	20 µl	2120 µl

[a]Multiply the number of samples in each run by 1.1 to add a 10% volume increase to account for pipet and human error (i.e., $96 \times 1.1 = 106$).

Table 15D.4.2 qRT-PCR Cycling Conditions for ZIKV Detection

Cycle	Temperature	Time (min:sec)	Repeats
1	50°C	30:00	1
2	95°C	15:00	1
3	94°C	00:15	40
	60°C	01:00	
4	4°C	indefinitely	hold

5. Add 5 µl RNA to each sample well; leave 12 wells free for controls.

   The authors recommend the use of a multichannel pipet for this step.

6. Add 5 µl of 10^{-1}, 10^{-2}, and 10^{-3} dilutions of previously titered and extracted positive control RNA and nuclease-free water to the appropriate wells.

7. Seal the optical well plate using the optical seal.

   Use the provided tool to press down the edges of the seal tightly to the plate. If the seal is not tight enough it can open during the reaction. This may cause samples to evaporate, leading to false positives.

8. Run one-step qRT-PCR using MyiQ Single-Color Real-Time PCR Detection System as shown in Table 15D.4.2.

   Any appropriately shaped curve that crosses the threshold is considered positive for ZIKV.

SUPPORT PROTOCOL 1

PROPAGATION OF VERO E6 CELLS

Vero E6 cells were originally derived from healthy kidney cells of the African green monkey (*Cercopithecus aethiops*). This specific cell line is a clone of the Vero 76 cell line. When Vero E6 cells reach confluency they will stop growing and begin to degrade (Ammerman et al., 2008). This is why it is important to monitor cell development and subculture before the cells reach 100% confluency. Cells are monitored daily and subcultured every 3 to 4 days. After dissociation, cell suspensions are either transferred to a new flask or seeded into 6-well plates. Keep record of the passage number. We recommend monitoring for bacterial contamination monthly by inoculating agar plates with swabs of culture media and using mycoplasma detection kits (e.g., Sigma, cat. no. MP0035-1KT). If bacterial contamination is detected, immediately dispose of the culture flask and begin sterilization of the BSC and cell culture incubator. Frozen stocks of low-passaged (passage number <50) Vero E6 cells are kept in liquid nitrogen at −191°C

with a final concentration of 10% dimethyl sulfoxide (DMSO) and 20% FBS. DMSO is a cryoprotectant added to cell culture media to decrease the formation of ice which reduces cell death during the freezing process. All procedures should be performed in a BSC with proper aseptic technique.

Materials

Vero E6 cell line (ATCC #CRL-1586)
DPBS (see recipe)
Vero E6 culture medium (see recipe)
0.25% trypsin/EDTA (e.g., Invitrogen, cat. no. 25200-072)

75-cm^2 tissue culture-treated flasks (sizes vary with personal needs)
Cell culture incubator set to 37°C and 5% CO_2
37°C water bath
Centrifuge

Media and reagent preparation

1. Begin with a 75-cm^2 flask that is ~80% to 90% confluent.
2. Warm DPBS, Vero E6 culture medium, and 0.25% trypsin/EDTA to 37°C in a water bath.

   All solutions that come into contact with the cells should be previously warmed to 37°C.

3. Remove media and rinse the flask with 5 to 10 ml of DPBS.

   Do not apply the DPBS with high pressure directly to the cells as this may cause the monolayer to disassociate from the culture flask. Gently add the DPBS down the neck of the flask and rock backward and forward and left to right for 1 to 3 min to aid in the removal of cellular debris and excess media.

Cell dissociation

4. Add 2 to 3 ml of 0.25% Trypsin/EDTA directly to the monolayer.

   Gently rock the flask backward and forward and left to right so that the trypsin covers the entire monolayer.

5. Incubate the flask at 37°C and 5% CO_2 for 2 to 4 min.

   Do not incubate for longer than 5 min.

6. After incubation, vigorously rock the flask from side to side.

   Check to see if the monolayer has been removed from the surface of the flask; the bottom of the flask should no longer be opaque.

7. Once the cells have disassociated from the flask immediately add 10 ml Vero E6 culture media to the flask.
8. Pipet up and down to vigorously dispense the cell-media solution where the monolayer used to be in order to dislodge any remaining cells.

   Repeat this step for 3 to 5 min to ensure all of the cells are suspended in solution. Be careful not to create excessive air bubbles.

9. Centrifuge the cell suspension 5 min at 200 × *g*, room temperature, to pellet the cells.
10. Remove and discard supernatant.

    Be careful not to disturb the pellet.

Cell resuspension and subculturing

11. Resuspend cells in 10 ml Vero E6 culture media.
12. For a 1:10 dilution add 1 ml of the cell suspension to a new 75-cm^2 flask.

    A 1:10 dilution should take 3 to 4 days to reach 80% to 90% confluence in a 75-cm^2 flask.

13. Add 10 to 15 ml of warmed Vero E6 culture media to the culture flask.
14. Incubate the culture flask at 37°C and 5% CO_2.
15. Monitor cell growth daily.
16. When cells reach an 80% to 90% confluent monolayer proceed to step 2.

SUPPORT PROTOCOL 2

PROPAGATION OF C6/36 CELLS

C6/36 cells were originally derived from larval tissue of *Aedes albopictus*. Cells are monitored daily and subcultured every 3 to 4 days. After dissociation, cell suspensions are either transferred to a new flask or seeded into well plates. Keep record of the passage number. We recommend monitoring for bacterial contamination monthly as described in Support Protocol 1. Frozen stocks of low passaged C6/36 cells are kept in liquid nitrogen at −191°C with a final concentration of 5% DMSO and 20% FBS. All procedures should be performed in a BSC with proper aseptic technique.

Materials

C6/36 cell line (ATCC #CRL-1660)
C6/36 culture medium (see recipe)
DPBS (see recipe)

75-cm^2 tissue culture-treated flasks (sizes vary with personal needs)
Cell culture incubator set to 28°C and 5% CO_2
28°C water bath
Cell scraper (sizes vary with personal needs)

1. Begin with a 75-cm^2 flask that is ~80% to 90% confluent.
2. Warm DPBS and C6/36 culture medium to 28°C in a water bath.

   All solutions that come into contact with the cells should be previously warmed to 28°C.

3. Remove media and rinse the flask with 5 to 10 ml DPBS.

   Do not apply the DPBS with high pressure directly to the cells as this may cause the monolayer to disassociate from the culture flask. Gently add the DPBS down the neck of the flask and rock backward and forward and left to right for 1 to 3 min to aid in the removal of cellular debris and excess media.

4. Scrape the cells with a cell scraper.

   Gently press the cell scraper to the monolayer and move along the entire surface area of the monolayer to remove the cells.

5. Resuspend the cells in 10 ml C6/36 culture medium.

   Mix thoroughly to avoid large clumps of cells.

6. Split the cells into a new 75-cm^2 culture flask at a 1:3 or 1:5 dilution.

   A 1:5 dilution should take 3 to 4 days to reach 80% to 90% confluency in a 75-cm^2 flask.

7. Add 10 to 15 ml of warmed C6/36 culture media to the culture flask.
8. Incubate the culture flasks at 28°C and 5% CO_2.
9. Monitor cell growth daily.

10. When cells reach an 80% to 90% confluent monolayer proceed to step 2.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A.

C6/36 culture medium

20 ml penicillin/streptomycin (e.g., ThermoFisher, cat. no. 15140122)
100 ml fetal bovine serum (FBS; e.g., Sigma, cat. no. F7942)
880 ml Minimum Essential Medium Eagle (MEME; e.g., Sigma, cat. no. M0643-10X1L)
Filter sterilize (0.2 μm) and store at 4°C for up to 4 to 6 weeks

Carboxymethylcellulose (CMC) solution

32 g powdered CMC
500 ml distilled water
Bring volume to 1 liter with distilled water
Mix with a magnetic stir bar at 100°C for 4 to 6 hr (ensuring it does not boil) until fully homogenized
Autoclave at 121°C for 10 min and store at room temperature for up to 6 months

Slowly add CMC powder to a vortex of agitated water or it will clump together or adhere to the bottom of the beaker.

CMC/DMEM overlay

Warm CMC solution (see recipe) and DMEM/2% FBS (see recipe) to 37°C. Combine equal volumes of CMC solution and DMEM/2% FBS. Mix with a sterile magnetic stir bar or with a 25-ml serological pipet. Store at 4°C for up to 4 to 6 weeks.

Crystal violet staining solution

10 g powdered crystal violet
300 ml 100% ethanol
200 ml formaldehyde
Bring final volume up to 1 liter with DPBS (see recipe)
Store in an amber flask at room temperature for 4 to 6 weeks

Dissolve crystal violet powder in ethanol first, and gently mix by rotation and gentle rocking to ensure the powder is fully dissolved.

After adding the formaldehyde and bringing the total volume to 1 liter with DPBS, stir with a magnetic stir bar for 30 min.

Keep out of direct sunlight.

Dulbecco's modified Eagle medium (DMEM)/2% FBS

20 ml fetal bovine serum (FBS)
980 ml DMEM (e.g., Sigma, cat. no. D6546)
Filter sterilize (0.2 μm) and store at 4°C for up to 4 to 6 weeks

Dulbecco's phosphate-buffered saline (DPBS)

Obtain a 1× DPBS solution (see *APPENDIX 2A* or purchase). Filter sterilize (0.2 μm) and autoclave at 121°C for 20 min. Store at room temperature for up to 6 months.

TNE

15 ml 5 M NaCl
5 ml 1 M Tris·Cl, pH 8.0 (see *APPENDIX 2A*)
1 ml 500 mM EDTA

continued

Bring final volume up to 500 ml with distilled water and stir with a magnetic stir bar for 30 min
Filter sterilize (0.2 μm) and store at 4°C for 4 to 6 weeks

TNE/25% glycerol

75 ml TNE (see recipe)
25 ml glycerol (e.g., Sigma, cat. no. G5516)
Stir with a magnetic stir bar for 10 min
Filter sterilize (0.2 μm) and store at 4°C for 4 to 6 weeks

Vero E6 culture medium

20 ml penicillin/streptomycin/L-glutamine (e.g., ThermoFisher, cat. no. 10378016)
100 ml fetal bovine serum (FBS)
880 ml Dulbecco's modified Eagle medium (DMEM)
Filter sterilize (0.2 μm) and store at 4°C for up to 4 to 6 weeks

COMMENTARY

Background Information

Quantification of ZIKV by plaque assay

Plaque assay has been considered the gold standard for detection and quantification of a wide variety of viruses. Modifications of the assay we describe here are suitable for other mammalian susceptible flaviviruses such as dengue and West Nile (plaque formation incubation periods vary). Other modifications may include choice of culture medium, final FBS concentration, and the addition of antibiotics or antifungal components.

Many researchers use an agar overlay during the plaque formation incubation period. Major drawbacks of this method are the temperature limitations when working with liquid agar. If the liquid agar is too hot it can damage the cells; if the liquid agar temperature drops below ~35°C it will begin to solidify. We use a 1:1 ratio of CMC and DMEM/2% FBS to overlay the infected cell monolayers instead of agar for these reasons. CMC is semi-solid at room temperature and can exhibit reduced viscosity when warmed to 37°C. Warming both CMC and DMEM/2% FBS to 37°C will reduce overlay preparation time.

ZIKV stock solutions were provided by the Public Health Agency of Canada and propagated and stored according to Brien et al. (2013).

Propagation of Zika virus in vitro

ZIKV can be easily propagated in Vero E6 and C6/36 cells. Propagation of virus in cell culture is performed to increase the viral titer and to maintain adequate volumes of low passage stock solutions.

Detection of ZIKV by qRT-PCR

Plaque assay is used to quantify live attenuated virus, but it does not identify which virus is causing the cytopathic effect in vitro. qRT-PCR is a quick and efficient method for detecting the presence of viral gene segments. We recommend using plaque assay to determine the PFU/ml of viral stocks and PCR screening to verify which virus is causing cytopathic effect.

Critical Parameters and Troubleshooting

We have found that ZIKV is easily propagated in the laboratory in cell culture. While performing the described protocols we have observed and recorded best practices that yield optimal results.

Quantification of ZIKV by plaque assay

It is important not to damage the cell monolayer by mechanical forces during media removal, viral inoculation, addition of the overlay, and subsequent washing with DPBS. During these processes be careful not to touch the monolayer. Leaning the well plate at an ~30° angle can aid in removing media and wash solutions. Pipet scratches can be observed in Figure 15D.4.2B, identified by the arrow.

If the CMC overlay is not appropriately removed by washing with DPBS, it can be difficult to observe plaque morphology and counts. We incubated a confluent 6-well plate for 3 days with a CMC/DMEM overlay and then washed with 0.5 ml DPBS (instead of 2 ml as described above) to highlight the effects of inadequate washing. The well that received 0.5 ml DPBS did not fully dissolve excess

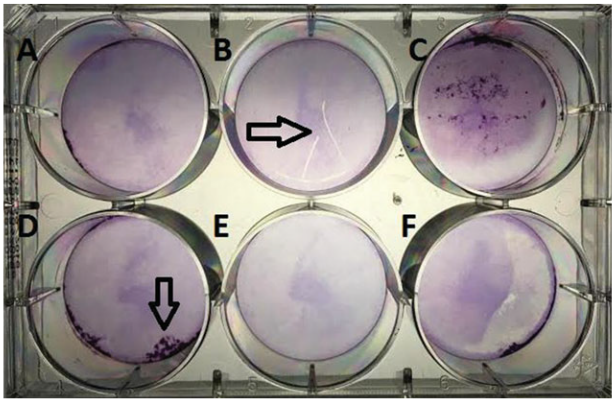

Figure 15D.4.2 Common human errors encountered during Basic Protocol 1. (**A**) Control well. (**B**) Mechanical damage by pipet scratches. (**C**) Using 0.5 ml DPBS to remove excess CMC/DMEM overlay as opposed to 2 ml. (**D**) Well was subjected to low water pressure during the poststain rinse. (**E**) Well was subjected to high water pressure during the poststain rinse. (**F**) Well was left on benchtop without any liquid to cover the monolayer for 30 min prior to Basic Protocol 1.

CMC/DMEM overlay (Fig. 15D.4.2C). Subsequently, any excess overlay will be stained by the crystal violet solution thereby producing patches of dark clumps that may interfere with plaque observation. Excess overlay can also adhere to the cells and remove small regions of the monolayer during the poststain wash (observed by the small crescent moon shaped area without cells). Using 2 ml of DPBS will ensure that an adequate amount of excess overlay in each well is dissolved. Once the DPBS is added the plates can be placed at 37°C to help facilitate this process.

After the staining process the well plates must be washed. To highlight the effects of inadequate washing we stained two confluent wells as outlined in Basic Protocol 1. The well in Figure 15D.4.2D was rinsed with a gentle or low water pressure, and the well in Figure 15D.4.2E was washed with a high water pressure. If the water pressure is too low excess overlay and staining solution will not be removed (noted by the arrow in Fig. 15D.4.2D). We observed that high water pressure works well to remove any excess overlay and staining solution and does not mechanically damage the monolayer or cause cells to dissociate with the plate. However, we did observe a lighter staining when compared to the control well.

The cells can dry out and subsequently die if not covered with any liquid media or DPBS. A well plate left for 30 min without any liquid overlay can be observed in Figure 15D.4.2F. The well was then covered with the CMC/DMEM overlay for 3 days and stained as described in Basic Protocol 1. As seen in Figure 15D.4.2F, the dried out cells became damaged and disassociated from the monolayer producing a large crescent moon shaped plaque. We recommend that the cells are not left without liquid overlay for more than 10 min. Work quickly and at your own pace to avoid drying out the monolayer. When working with well plates, minimize the amount of time the lid is open to reduce cell exposure.

To determine the optimal overlay incubation time for observation and counting of ZIKV plaques, we performed plaque assays with various overlay incubation periods in quadruplet. Cytopathic effect can be observed as early as 3 days postinfection. A 5 day overlay incubation period produces medium sized plaques that are optimal for counting (Fig. 15D.4.3). A 6 day overlay incubation also produced plaques adequate for counting; however, in some instances a 6 day overlay incubation period yielded plaques so large that two or three adjacent plaques became combined, which may cause difficulties with counting plaques (Fig. 15D.4.4). Based on our observations, we recommend a 5 day overlay incubation period for optimal plaque observation.

Generation and purification of ZIKV stocks

Generating usable virus stocks is dependent on the qualities of both the cells used for propagation and the virus itself. Initial infection using cells at a high confluency (>90%) with a low (0.01 to 0.5) MOI ensures adequate production of infective virus. While the usage of cells that have undergone multiple passages has been observed to have little to no effect on the outcome of viral propagation, the use of virus that has been serially passaged multiple

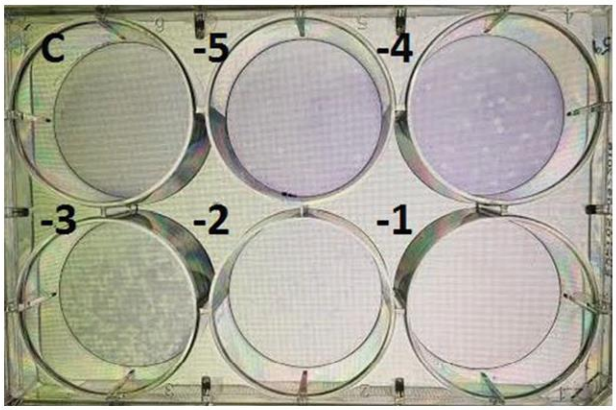

Figure 15D.4.3 Zika virus plaque assay 5 day postinfection incubation period. C = control. The −4 dilution (top right) demonstrates the ideal plaque size for counting.

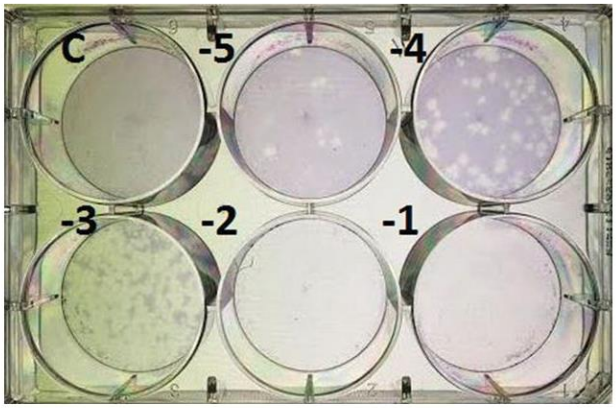

Figure 15D.4.4 Zika virus plaque assay 6 day postinfection incubation period. C = control. The −4 dilution (top right) contains the required 30 to 100 plaques for ideal plaque counting. However, due to the additional 24 hr incubation period (when compared to the 5 day trial) many adjacent plaques have merged together creating numerous large plaques. Since the number of plaques that make up these large plaques is unclear, we suggest postinfection incubation periods of no longer than 5 days for optimal plaque counts.

times in vitro should be avoided. Due to the accumulation of genetic mutations over time it is recommended that a single parental unpurified virus stock is generated which can then be used to create subsequent virus stocks to be used for experiments.

Basic Protocol 2 describes virus stock purification through a glycerol cushion. Plaque purification and density gradient centrifugation (Unit 15D.2; Medina et al., 2012) can also be performed to increase the concentration and eliminate nonvirulent virions from stock solutions.

Detection of ZIKV by qRT-PCR

It is common to encounter nonspecific amplification or probe degradation ("noise") with higher cycle numbers, such as those around or above 40. Samples with a cycle threshold (Ct) value above any negative control signal must be deemed negative. With a PCR efficiency of 98.4% (slope −3.36, $R^2 = 0.9924$), we observed that reaction saturation typically occurs by cycle 35, and thus we consider any signal above 37 a negative result. As PCR efficiency can vary in and between laboratories, the cutoff for a positive Ct value must be determined by using the standard curve to estimate the lower limit of detection of the assay. This is why we include known standards, in triplicate, in every reaction setup (Caraguel et al., 2011).

Anticipated Results

Quantification of ZIKV by plaque assay

Basic Protocol 1 is an efficient way to quantify PFU/ml of viral stock solutions and sam-

ples that contain ZIKV infected animal tissues. Plaques can be observed as early as 3 days postinfection. For best results we recommend a postinfection incubation period of 5 days (Fig. 15D.4.3). A 6-day postinfection period produced large plaques that proved difficult to count as adjacent plaques become conjoined (Fig. 15D.4.4). If the titer of your sample is very large ($>10^7$ PFU/ml), the 10^{-5} dilution may have more than 100 plaques. We suggest repeating this protocol with lower serial dilutions until a dilution produces the desired number of plaques for counting, namely 30 to 100 plaques.

Generation and purification of ZIKV stocks

Generally, nonpurified ZIKV stocks derived from Vero cells will reach titers of $\sim 10^6$ PFU/ml and 10^7 PFU/ml from C6/36 cells. Purification following the protocols outlined in *UNIT 15D.3* (Brien et al., 2013) can increase titer to $\sim 10^7$ with Vero cells and 10^8 with C6/36 cells.

Time Considerations

Quantification of ZIKV by plaque assay

This titration assay takes a total of 5 to 6 days. A number of solutions and reagents require additional preparation time. Well plates are to be seeded 2 to 3 days before Basic Protocol 1 is initiated as the cells require time to adhere to the well plates and replicate to the desired confluency. The CMC solution should be prepared 1 to 2 days before plaque assay is attempted as it can take up to 6 hr for the CMC powder to dissolve in water. This time is dependent on the final solution volume; the estimated times are for a final volume of 1 liter. The 3.2% CMC solution must then be autoclaved and cooled to 37°C.

Preparation time for Basic Protocol 1 on the day plaque assay is attempted can take 1 to 2 hr. It is important to schedule adequate time for the reagents to warm to 37°C. Additional time is required to prepare the desired dilutions of the samples to be tested and well plate preparation.

On the day of staining, minimal preparation is required. We recommend that the wells be stained with the crystal violet solution for at least 30 min. Turn the lights off in the BSC to reduce degradation of the crystal violet solution. After the crystal violet solution has been removed and rinsed in the sink, invert the plates and dry for 4 to 6 hr or overnight before counting plaques.

Generation and purification of ZIKV stocks

Preparation time for Basic Protocol 2 can take 3 to 4 days as 90% to 95% confluent 75-cm^2 flasks are required. Previously titered ZIKV stock solutions must be prepared well before attempting this protocol. It is recommended to first create one large virus stock with a high titer ($>10^5$ to 10^7 PFU/ml) as it can be used repeatedly for many experiments and diluted if needed. Cytopathic effect is generally observed 40 to 48 hr postinfection; this is dependent on MOI and cell strain.

Detection of ZIKV by qRT-PCR

Preparation time for Basic Protocol 3 can take 30 to 60 min as reagents require time to thaw on ice. Loading the wells with master mix and RNA can be time consuming. We recommend purchasing a multichannel pipet when testing a large number of samples. The PCR reaction takes ~2 hr.

Propagation of Vero E6 cells

It typically takes two to three passages from frozen stock to reach regular growth (approximately doubling every 24 hr; Ammerman et al., 2008). Preparation time for Support Protocol 1 can take 30 to 60 min. It is important to schedule adequate time for all cell culture reagents to warm to 37°C prior to coming into contact with the cells. Vero E6 cells should be passaged every 3 to 4 days or before reaching 80% to 90% confluency.

Propagation of C6/36 cells

Preparation time for Support Protocol 2 can take 30 to 60 min. It is important to schedule adequate time for all cell culture reagents to warm to 28°C prior to coming into contact with the cells. C6/36 cells should be passaged every 3 to 4 days or before reaching 80% to 90% confluency.

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Conflict of Interest

The authors declare no conflict of interest.

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Chapter 4

The Vector Competence of Canadian Mosquitoes to Zika Virus

4.1 Abstract

Zika Virus (ZIKV), belonging to the family Flaviviridae, genus *Flavivirus*, is an arthropod-borne virus that has been implicated in inducing teratogenic effects and neurodegenerative disease in humans. First discovered in Uganda in 1947, it had slowly spread throughout Southeast Asia before being detected again in Brazil in 2014. Spread by mosquitoes, ZIKV has rapidly spread throughout the Americas, with multiple species being implicated as vectors. Most positive cases of Zika in Canada are travel related and some sexually transmitted, with no locally acquired cases being reported. Whether or not Canadian mosquitoes are able to spread Zika virus remains unanswered. By orally infecting wild caught mosquitoes with a ZIKV infected sugar meal and detecting the presence of virus 10 and 14 days post infection (d.p.i.), the vector competence of Canadian mosquitoes was evaluated. It was found that after 10 (n=50) and 14 d.p.i. (n=32), 2% and 0% of a population *of Culex pipiens* mosquitoes were found to be able to become infected and transmit the virus, respectively. Although *Culex pipiens* mosquitoes from the Niagara region may not be vectors of ZIKV, that does not negate other Canadian mosquitoes as being potential vectors.

4.2 Introduction

Zika virus (ZIKV; family Flaviviridae, genus *Flavivirus*) is an enveloped, positive-sense, single-stranded RNA virus that is transmitted to humans primarily by mosquitoes. Originally discovered in the forests of Uganda (Dick *et al*., 1952), ZIKV was largely ignored due to its rarity and non-fatal induced symptoms. Endemic to Africa, ZIKV spread throughout Asia eventually reaching Southeast Asian peninsulas (Jan *et al*., 1978; Olsen and Ksiazek, 1981; Robin *et al*., 1975). Outbreaks in Yap Island and the French Polynesian Islands prompted

increased surveillance of ZIKV as the severity of symptoms and frequency of human infection were slowly increasing (Duffy *et al.,* 2009). ZIKV successfully traversed to the Western hemisphere sometime in 2014, initially being found in Brazil (Campos *et al*., 2015; Zanluca *et al.,* 2015). Recently, ZIKV has spread throughout the Americas causing panic due to the perceived threat that it was able to induce both teratogenic effects and neurodegenerative disease. The rapid spread of ZIKV is arguably due to local and invasive mosquito species being competent vectors. Like most mosquito associated flaviviruses, viral replication is dependent on the mosquito host. If permissive to infection, ZIKV can disseminate to the salivary glands of a mosquito where it can be transmitted during the next blood feeding. The origin and early spread of ZIKV throughout Africa and Asia has largely implicated *Aedes* mosquitoes as the primary vectors (Macnamara, 1954; Marchette *et al*., 1969) so research has disproportionately been focused on the two most common *Aedes* species: *Aedes aegypti* and *Aedes albopictus*. While found throughout the Americas, the two are largely contested to be the only vectors of ZIKV. As ZIKV spreads throughout the Americas, Canada has yet to establish whether native populations of mosquitoes are competent vectors. To determine the vector competency of Canadian mosquitoes with regards to ZIKV, local mosquito populations need to be caught and infected. We analyzed the vector competency of Canadian mosquitoes by orally introducing virus through an infective sugar meal and tested for its presence 10 and 14 days post infection (d.p.i.) with qRT-PCR. Understanding which mosquito species are implicated in transmitting ZIKV will help better prepare Canada's response. More accurate forecasting models can be made that will better predict when and where outbreaks will occur. Knowing which species are implicated will also allow better targeted control efforts to help reduce the likelihood of spreading.

4.3 Methods

4.3.1 Collection and Rearing

Wild *Culex* mosquitoes were collected 4 days a week during the month of August 2016 from 9 collection sites within the Niagara region (Appendix). Both mosquito larvae/pupae and *Culex egg* rafts were collected using a larval dipper (BioQuip). Mosquito eggs and larvae were reared to adulthood in standard conditions (See Chapter Two).

4.3.2 Virus

The Thai 2013 ZIKV strain obtained from The National Microbiology Laboratory (Winnipeg, MB, CAN) on behalf of the Public Health Agency of Canada, was used to infect the mosquitoes. The viral strain was derived from the urine of a female tourist visiting Thailand (Fonseca *et al*., 2014). It was later isolated and passaged 5 times (p5) in Vero E6 cell line (p43) and suspended with 20% fetal bovine serum (FBS). Stock titre used was 10^6 PFU.

4.3.3 Oral Infection of Mosquitoes

Prior to infection, adult mosquitoes were deprived and starved for 24hrs from both water and sucrose solutions. Approximately 3 to 5 day old female mosquitoes (n=120) were sorted, split and transferred into 3 sealed 0.25 L containers. Cotton pads soaked with a viral sugar solution (Appendix) were placed on top of each container for the mosquitoes to feed on. The solution was pre-warmed to 37 degrees Celsius before saturating the cotton pad. Mosquitoes fed for 30 minutes before being cold anaesthetized. Fully engorged females were collected and transferred to new 0.25 L sealed containers and 28°C ± 1°C with a photoperiod of 16h:8h light:

dark cycle at 75% ± 5 % humidity. Mosquitoes were provided with 10% sucrose soaked cotton pads changed daily (Appendix). All experiments were carried out in a containment level 3 (CL3) facility.

4.3.4 Mosquito Processing

To determine ZIKV infection, dissemination, and transmission rates, mosquitoes were dissected after 10 and 14 days post infection (dpi). Surviving orally infected mosquitoes were cold anaesthetized, individually sorted, and identified to species individually using a photographic key (Thielman and Hunter, 2007). Legs and wings were removed and suspended in 500 µL of mosquito diluent solution (Appendix). Saliva was then collected from the amputated mosquito as previously described with modification (Anderson *et al*., 2010). The proboscis of the mosquito was inserted into a capillary tube containing 5 µL of mosquito saliva collection solution (Appendix). Approximately 1 µL of a 1% pilocarpine solution being applied to the thorax to induce salivation (Dubrulle et al., 2009). After 30 minutes of salivation, the mosquito was removed from the capillary tube and the body suspended in 500 µL of mosquito diluent solution. The contents of the capillary tube post salivation were expelled and collected in 300 µL of mosquito diluent solution. The process was repeated for each individual mosquito. Mosquito body and leg/wing samples were later homogenized for RNA extraction (Appendix). To test the validity of the feeding method, newly fed mosquitoes (0 d.p.i.) (n=13) were sorted and frozen at -80 degrees Celsius in 500 µL of mosquito diluent solution. Their whole body was later homogenized for RNA extraction.

4.3.5 Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) assay

Total RNA was isolated from body, leg/wing, and saliva samples using the Total RNA Purification Kit (Norgen, Canada) following manufacturer's recommendations. ZIKV in all 3 samples was detected using a one-step real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) as previously described with modification (Agbulos *et* al., 2017; Lancotti *et al*., 2008).

4.3.6 Data Analysis

Each mosquito yielded 3 samples: the body, leg/wing, and saliva. Infection rate (IR) was determined by the percentage of ZIKV positive body samples. Dissemination rate (DR) was then determined by the percentage of ZIKV positive leg/wing samples derived from mosquitoes whose bodies tested positive. Transmission rate was determined by the percentage of ZIKV positive saliva samples derived from mosquitoes whose body and leg/wing tested positive. Transmission efficiency (TE) was determined by the percentage of mosquitoes that tested positive for all 3 samples. Mosquitoes could transmit the virus only if it tested positive sequentially from body, to leg/wing, to finally saliva. Mosquito body samples that tested negative, negated the result of the other two samples, omitting it from further analysis.

4.4 Results

Female mosquitoes used in the experiment were identified to be exclusively *Culex pipiens*. All 0 d.p.i. mosquitoes tested positive for ZIKV (Appendix). After 10 d.p.i., 50 mosquitoes were dissected and subjected to qRT-PCR. Out of the 50 mosquitoes, 7 mosquito bodies tested positive (IR=14.00%) for ZIKV (Table 1). From the 7, only 1 leg/wing sample

tested positive (DR=14.29%) for ZIKV. The lone mosquito whose body and leg/wing samples that tested positive for ZIKV also tested positive for its saliva sample (TR=100%). Out of the original 50 mosquitoes, only 1 tested positive for all three (TE=2.00%)

After 14 d.p.i., 32 mosquitoes were dissected and subjected to qRT-PCR. Out of the 32 mosquitoes, 1 mosquito body tested positive (IR=3.10%) for ZIKV (Table 1). The lone mosquito tested negative for both leg/wing and saliva samples (DR and TR=0%). Out of 32 mosquitoes, none had disseminated nor transmitted the virus (TE=0%)

Table 1. Infection, dissemination, transmission rates and efficiency for *Culex pipiens molestus* orally fed ZIKV and reared at 28°C at 10 and 14 days post infection (d.p.i.*).* n= 50 after 10 d.p.i. and *n*= 32 after 14 d.p.i. Two tailed Fisher exact test used.

	10 d.p.i.	14 d.p.i.	p
Infection Rate (IR)	14.00% (7/50)	3.10% (1/32)	0.1409
Dissemination Rate (DR)	14.29% (1/7)	0% (0/1)	1.000
Transmission Rate (TR)	100% (1/1)	0%	1.000
Transmission Efficiency (TE)	2.00% (1/50)	0%	1.000

4.5 Discussion

Our goal was to determine the vector competence of Canadian mosquitoes to ZIKV. Our results suggest that female *Culex pipiens* mosquitoes from the Niagara region are poor vectors of ZIKV. We showed that after both 10 and 14 d.p.i., the IR, DR, TR, and TE were either low or non-existent. Low IRs after 10 and 14 d.p.i. limited the likelihood that the virus was capable of progressing to dissemination and transmission (Table 1). It appeared that longer incubation periods in the mosquitoes resulted in reduced vector competency as 14 d.p.i. mosquitoes had no

DR, TR and TEs while 10 d.p.i. mosquitoes had a higher IR, suggesting the clearance of virus from the mosquito host due to the mosquito immune response (Guo *et al.,* 2016; Salazar *et al.,* 2007).

Similar findings using laboratory colony and wild caught *Cx. pipiens, Cx. tarsalis* and *Cx. quinquefasciatus* mosquitoes suggest that *Culex* mosquitoes are poor vectors in general (Aliota *et al*., 2016; Amraoui *et al*., 2016; Boccolini *et al*., 2016; *Fernandes et al*., 2016; Huang *et al*., 2016; Weger-Lucarelli *et al*., 2016). Their generally ornithophilic feeding behaviour (Magnarelli, 1977) paired with the association of *Aedes* mosquitoes and ZIKV furthers the assumption. While few, there also exists conflicting evidence suggesting that *Cx. quinquefasciatus* is a capable vector (Guedes *et al*., 2016, Guo *et al.,* 2016). Not to be excluded, *Culex* mosquitoes have been hypothesized to be implicated in the transmission of ZIKA in some capacity. The reassignment of ZIKV from hemorrhagic/ *Aedes*-associated virus to neurotrophic/ *Culex*-associated virus increases the possibility that one or more unknown *Culex* species may be a vector (Leal, 2016). Recent forecasting models even predict other lesser known *Culex* species to play a role in transmission (Evans *et al*., 2016). Ambiguity in the vector competence of commonly tested *Culex* species hints at the possibility of innate differences between laboratory colonized and wild caught mosquito populations that dictate permissiveness to ZIKV infection. Observed in both laboratory and wild mosquito populations with various flaviviruses, these innate differences have been linked to genetics and/ or the presence of pathogens within the mosquito interfering with infection (Bolling *et al.,* 2015; Gubler *et al.,* 1979; Hardy *et al.,* 1976).

Discrepancies in vector competency status may also arise from differences in the virus used. The spread of ZIKV from Uganda to the Americas has produced numerous strains which have been employed by many researchers which have also been fed to mosquitoes in various

concentrations (Aliota *et al*., 2016; Amraoui *et al*., 2016; Boccolini *et al*., 2016; Fernandes *et al*., 2016; Guedes *et al*., 2016, Guo *et al.,* 2016; Huang *et al*., 2016; Weger-Lucarelli *et al*., 2016). Differences in virulence has been noted between strains both *in vivo* and *in vitro* (Simionin *et al.,* 2016; Stauft *et al*., 2016. Weger-Lucarelli *et al*., 2016). Although this study could not successfully infect mosquitoes as others have, the similar strains of virus and concentrations used have previously been used to successfully infect (Guedes *et al*., 2016, Guo *et al.,* 2016).

The method of feeding used in this study may have altered the results; compared to every other study in which mosquitoes have been infected with ZIKV suspended in blood, this study used ZIKA in a sucrose meal. Aragão (1922) first successfully infected male *Aedes aegypti* mosquitoes with YFV in a honey/ blood suspension when it was only assumed that females could be infected. Proof of concept showed that virus was found 0 d.p.i. in whole mosquitoes and that infected bodies were still found 10 and 14 d.p.i suggesting its usefulness in some capacity. WNV had been previously used to successfully infect both *Aedes aegypti* and *Aedes albopictus* in similar fashion further suggesting its use as a viable alternative to blood (Causarano, 2017).

Since there is no consensus from the scientific community on the vector competence status of mosquitoes and ZIKV, these findings only contribute to the growing need for future research. It is widely assumed that the principle vectors of ZIKV are mosquitoes belonging to the *Aedes* genus, subgenus Stegomyia, with emphasis on *Ae. aegypti* and *Ae. albopictus,* although other studies indicate that species from other genera most likely play some role. Although *Culex pipiens* mosquitoes from the Niagara region may not be good vectors of ZIKV, that does not negate other Canadian mosquitoes. With the recent arrival of *Ae. aegypti* and *Ae. albopictus* in Canada, the vector competence status of native species may become more complicated.

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Appendix

Recipes

Larval Feed

Mix 1:1 Brewer's yeast and crushed Tetramin™ fish food with pestle and mortar and store in sealed container in cooled, dry place.

10% Sucrose Solution

For 100 mL, weigh 10 grams of sucrose and pour into 70 mL of tap water. Mix and then fill to 100 mL. Autoclave and store in refrigerator.

SDS PAGE Sample Buffer

Mix 1.0 mL of 0.5mM Tris-HCl with pH 6.8, 2.0 ml of 25% glycerol, 0.08 mL of 1.0% bromophenol blue, 1.6 mL of 10% SDS, and 2.92 mL of deionized water, Add 0.4 mL of β-mercaptoethanol immediately before use.

Viral Sugar Solution

Mix 4.65ml of Dulbecco's Modified Eagle Medium High Glucose (DMEM), 0.75ml of 40% sucrose solution, and 0.6ml of 10^6 PFU ZIKV. Final concentration of virus is 10^5 PFU. Solution is warmed to 37°C before use.

Mosquito Diluent Solution

To make 100 mL, add 2 mL of FBS to 98 mL of DMEM. Keep refrigerated.

Mosquito Saliva Collection Solution

For 100 mL, mix 50 mL of 10% sucrose solution with 50 mL stock FBS solution.

Chapter 4 Data

MyiQ™ BIO-RAD

PCR Quantification Report
PCR Base Line Subtracted Curve Fit Data

Current Date:	**19-Sep-16 10:47 AM**
Data generated on:	**16-Sep-16 at 12:38 AM**.
Optical data file name:	**zika 10dpi culex sept 16.odm**
Plate Setup file used:	**sept15zika.psm**
Protocol file used:	**ZIKA.tmo**
Sample volume:	**25.00 ul**
Hot Start?	**No**
Well factor collection:	**Experimental Plate**

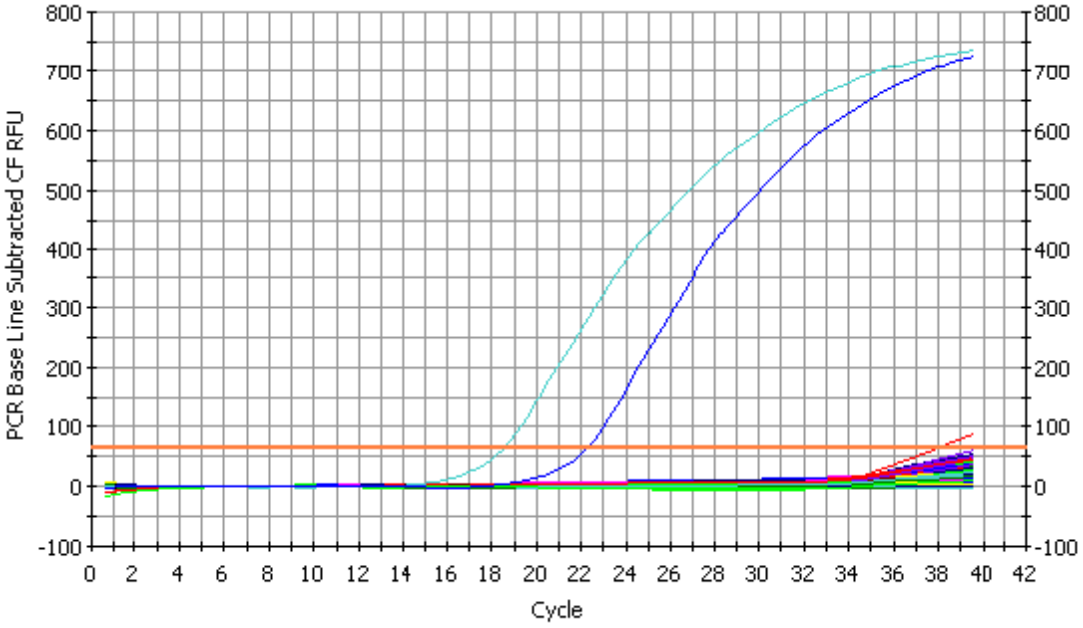
Comments

Protocol

Cycle 1: (1X)			
	Step 1:	**50.0ºC**	**for 30:00**
Cycle 2: (1X)			
	Step 1:	**95.0ºC**	**for 15:00**

Cycle 3: (40X)
Step 1: 94.0ºC for 00:15
Step 2: 60.0ºC for 01:00
Data collection and real-time analysis enabled.
Cycle 4: (1X)
Step 1: 4.0ºC HOLD

PCR Amp/Cycle Graph for FAM-490



Data Analysis Parameters

Calculated threshold has been replaced by the user selected threshold **63.9**.
Per-well baseline cycles have been determined automatically.
Data analysis window is set at **95.00%** of a cycle, centered at **end** of the cycle.
Weighted Mean digital filtering has been applied. Global filtering is **off**.

PCR Quantification Spreadsheet Data for FAM-490

Well	Identifier	Ct	Setpoint
A01		N/A	
A02		N/A	
A03		N/A	
A04		N/A	
A05		N/A	
A06		N/A	
A07		N/A	
A08		N/A	
A09		N/A	
A10		N/A	
A11		N/A	
A12		N/A	
B01		N/A	

B02	N/A
B03	N/A
B04	N/A
B05	N/A
B06	N/A
B07	N/A
B08	N/A
B09	N/A
B10	N/A
B11	N/A
B12	N/A
C01	N/A
C02	N/A
C03	N/A
C04	N/A
C05	N/A
C06	N/A
C07	N/A
C08	N/A
C09	N/A
C10	N/A
C11	N/A
C12	N/A
D01	N/A
D02	N/A
D03	N/A
D04	N/A
D05	N/A
D06	N/A
D07	N/A
D08	N/A
D09	N/A
D10	N/A
D11	N/A
D12	N/A
E01	N/A
E02	N/A
E03	N/A
E04	N/A
E05	N/A
E06	N/A
E07	N/A
E08	N/A
E09	N/A
E10	N/A
E11	N/A
E12	N/A
F01	N/A
F02	N/A
F03	N/A
F04	N/A
F05	N/A
F06	N/A
F07	N/A
F08	N/A
F09	N/A

F10	N/A
F11	N/A
F12	N/A
G01	N/A
G02	N/A
G03	N/A
G04	N/A
G05	N/A
G06	N/A
G07	N/A
G08	N/A
G09	38.01
G10	N/A
G11	N/A
G12	N/A
H07	N/A
H08	N/A
H10	18.59
H11	22.21

Wells Excluded from Analysis

A total of 8 well(s) have been excluded from analysis.

H01: <no identifier>	**H02: <no identifier>**	**H03: <no identifier>**
H04: <no identifier>	**H05: <no identifier>**	**H06: <no identifier>**
H09: <no identifier>	**H12: <no identifier>**	

Modified Well Contents

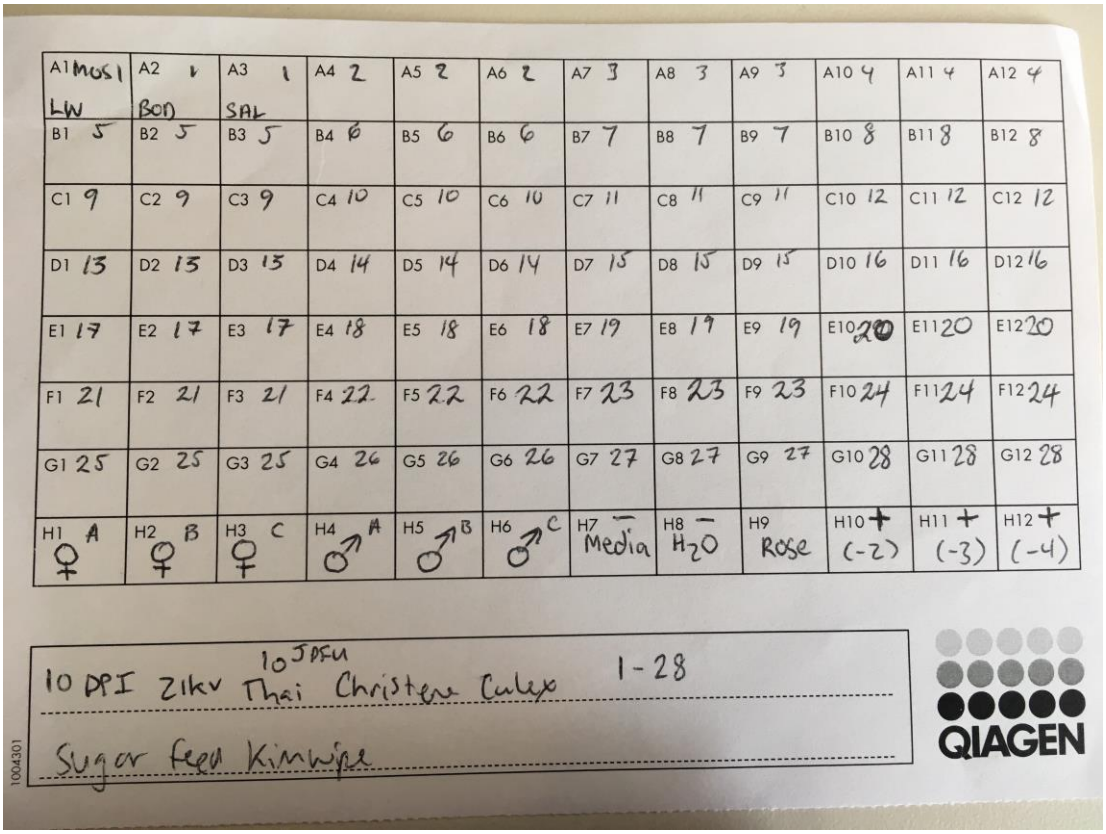
No wells have been modified.

MyiQ™ BIO-RAD

PCR Quantification Report
PCR Base Line Subtracted Curve Fit Data

Current Date:	**19-Sep-16 10:51 AM**
Data generated on:	**16-Sep-16 at 03:40 AM**.
Optical data file name:	**zika 10dpi culex 2 sept 16.odm**
Plate Setup file used:	**sept16.psm**
Protocol file used:	**ZIKA.tmo**
Sample volume:	**25.00 ul**
Hot Start?	**No**
Well factor collection:	**Experimental Plate**

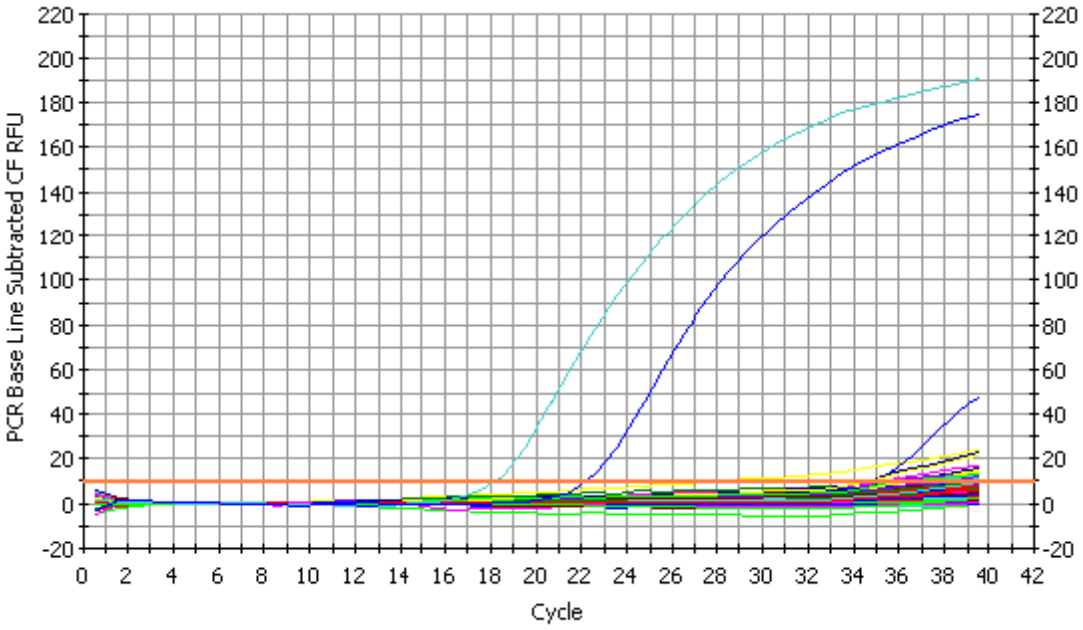
Comments



Protocol

Cycle 1: (1X)			
	Step 1:	**50.0ºC**	**for 30:00**
Cycle 2: (1X)			
	Step 1:	**95.0ºC**	**for 15:00**
Cycle 3: (40X)			
	Step 1:	**94.0ºC**	**for 00:15**
	Step 2:	**60.0ºC**	**for 01:00**
	Data collection and real-time analysis enabled.		
Cycle 4: (1X)			
	Step 1:	**4.0ºC**	**HOLD**

PCR Amp/Cycle Graph for FAM-490



Data Analysis Parameters

Calculated threshold using the **maximum curvature approach** is **9.2**.
Per-well baseline cycles have been determined automatically.
Data analysis window is set at **95.00%** of a cycle, centered at **end** of the cycle.
Weighted Mean digital filtering has been applied. Global filtering is **off**.

PCR Quantification Spreadsheet Data for FAM-490

Well	Identifier	Ct	Setpoint
A02		N/A	
A03		N/A	
A04		N/A	
A05		N/A	
A06		N/A	

A07	N/A
A08	36.01
A09	35.13
A10	27.37
A11	N/A
B01	N/A
B02	N/A
B03	35.54
B04	N/A
B05	N/A
B06	N/A
B07	N/A
B08	N/A
B09	N/A
B10	N/A
B11	35.22
B12	36.19
C01	35.07
C02	N/A
C03	37.24
C04	N/A
C05	N/A
C06	N/A
C07	N/A
C08	37.42
C09	N/A
C10	N/A
C11	N/A
C12	37.51
D01	N/A
D02	N/A
D03	N/A
D04	36.70
D05	N/A
D06	37.08
D07	N/A
D08	36.99
D09	N/A
D10	34.99
D11	N/A
D12	35.86
E01	N/A
E02	36.28
E03	N/A
E04	N/A
E05	N/A
E06	N/A
E07	N/A
E08	N/A
E09	N/A
E10	34.56
E11	37.71
E12	34.49
F01	N/A
F02	35.67
F03	N/A

F04	N/A
F05	N/A
F06	N/A
H07	N/A
H08	N/A
H10	18.08
H11	22.05

Wells Excluded from Analysis

A total of 28 well(s) have been excluded from analysis.

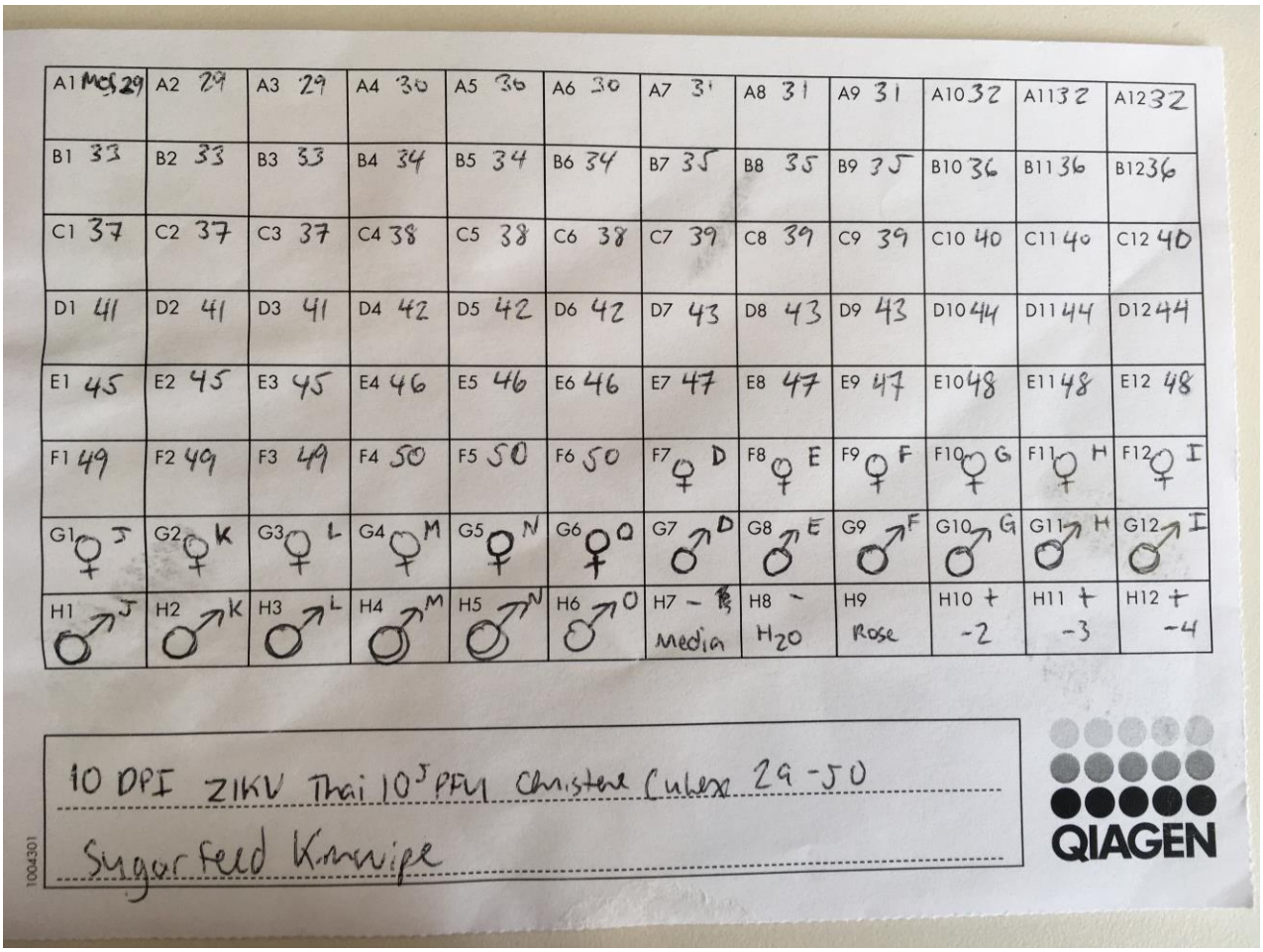
A01: <no identifier>	**A12: <no identifier>**	**F07: <no identifier>**
F08: <no identifier>	**F09: <no identifier>**	**F10: <no identifier>**
F11: <no identifier>	**F12: <no identifier>**	**G01: <no identifier>**
G02: <no identifier>	**G03: <no identifier>**	**G04: <no identifier>**
G05: <no identifier>	**G06: <no identifier>**	**G07: <no identifier>**
G08: <no identifier>	**G09: <no identifier>**	**G10: <no identifier>**
G11: <no identifier>	**G12: <no identifier>**	**H01: <no identifier>**
H02: <no identifier>	**H03: <no identifier>**	**H04: <no identifier>**
H05: <no identifier>	**H06: <no identifier>**	**H09: <no identifier>**
H12: <no identifier>		

Modified Well Contents

No wells have been modified.

MyiQ™ BIO-RAD

PCR Quantification Report
PCR Base Line Subtracted Curve Fit Data



Current Date:	**19-Sep-16 10:53 AM**
Data generated on:	**16-Sep-16 at 06:18 PM**.
Optical data file name:	**zika 14dpi culex sept 16.odm**
Plate Setup file used:	**sept16-14dpi.psm**
Protocol file used:	**ZIKA.tmo**

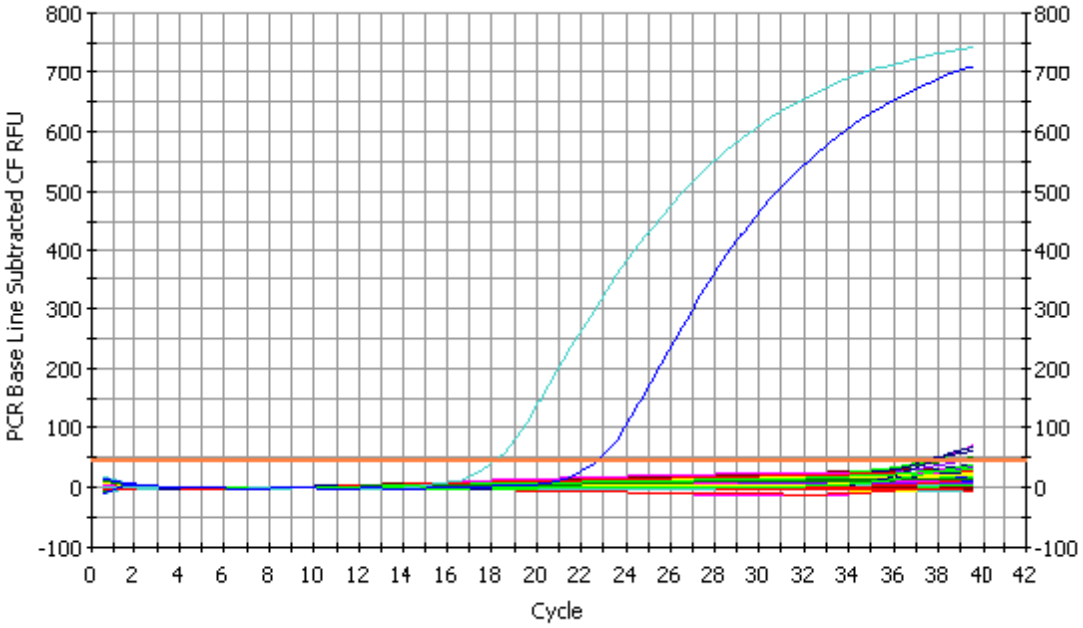
Sample volume: **25.00 ul**
Hot Start? **No**
Well factor collection: **Experimental Plate**

Comments

Protocol

Cycle 1: (1X)			
	Step 1:	**50.0ºC**	**for 30:00**
Cycle 2: (1X)			
	Step 1:	**95.0ºC**	**for 15:00**
Cycle 3: (40X)			
	Step 1:	**94.0ºC**	**for 00:15**
	Step 2:	**60.0ºC**	**for 01:00**
	Data collection and real-time analysis enabled.		
Cycle 4: (1X)			
	Step 1:	**4.0ºC**	**HOLD**

PCR Amp/Cycle Graph for FAM-490



Data Analysis Parameters

Calculated threshold has been replaced by the user selected threshold **43.9**.
Per-well baseline cycles have been determined automatically.
Data analysis window is set at **95.00%** of a cycle, centered at **end** of the cycle.
Weighted Mean digital filtering has been applied. Global filtering is **off**.

PCR Quantification Spreadsheet Data for FAM-490

Well	Identifier	Ct	Setpoint
A02		N/A	
A03		N/A	
A04		N/A	
A05		N/A	
A06		N/A	
A07		N/A	
A08		N/A	
A09		N/A	
A10		N/A	
A11		N/A	
B01		N/A	
B02		N/A	
B03		N/A	
B04		37.80	
B05		N/A	
B06		N/A	
B07		N/A	
B08		N/A	
B09		N/A	
B10		37.38	
B11		38.25	
B12		N/A	
C01		N/A	
C02		N/A	
C03		N/A	
C04		N/A	
C05		N/A	
C06		N/A	
C07		N/A	
C08		N/A	
C09		N/A	
C10		N/A	
C11		N/A	
C12		N/A	
D01		N/A	
D02		N/A	
D03		N/A	
D04		N/A	
D05		N/A	
D06		N/A	
D07		N/A	
D08		N/A	
D09		N/A	
D10		N/A	
D11		N/A	
D12		N/A	
E01		N/A	
E02		N/A	
E03		N/A	
E04		N/A	
E05		N/A	
E06		N/A	

E07	N/A
E08	N/A
E09	N/A
E10	37.39
E11	N/A
E12	N/A
F01	N/A
F02	N/A
F03	N/A
F04	N/A
F05	N/A
F06	N/A
F07	N/A
F08	N/A
F09	N/A
F10	N/A
F11	N/A
F12	N/A
G01	N/A
G02	N/A
G03	N/A
G04	N/A
G05	N/A
G06	37.69
G07	N/A
G08	N/A
G09	N/A
G10	N/A
G11	N/A
G12	N/A
H07	N/A
H08	N/A
H10	18.14
H11	22.68

Wells Excluded from Analysis

A total of 10 well(s) have been excluded from analysis.

A01: <no identifier>	**A12: <no identifier>**	**H01: <no identifier>**
H02: <no identifier>	**H03: <no identifier>**	**H04: <no identifier>**
H05: <no identifier>	**H06: <no identifier>**	**H09: <no identifier>**
H12: <no identifier>		

Modified Well Contents

No wells have been modified.

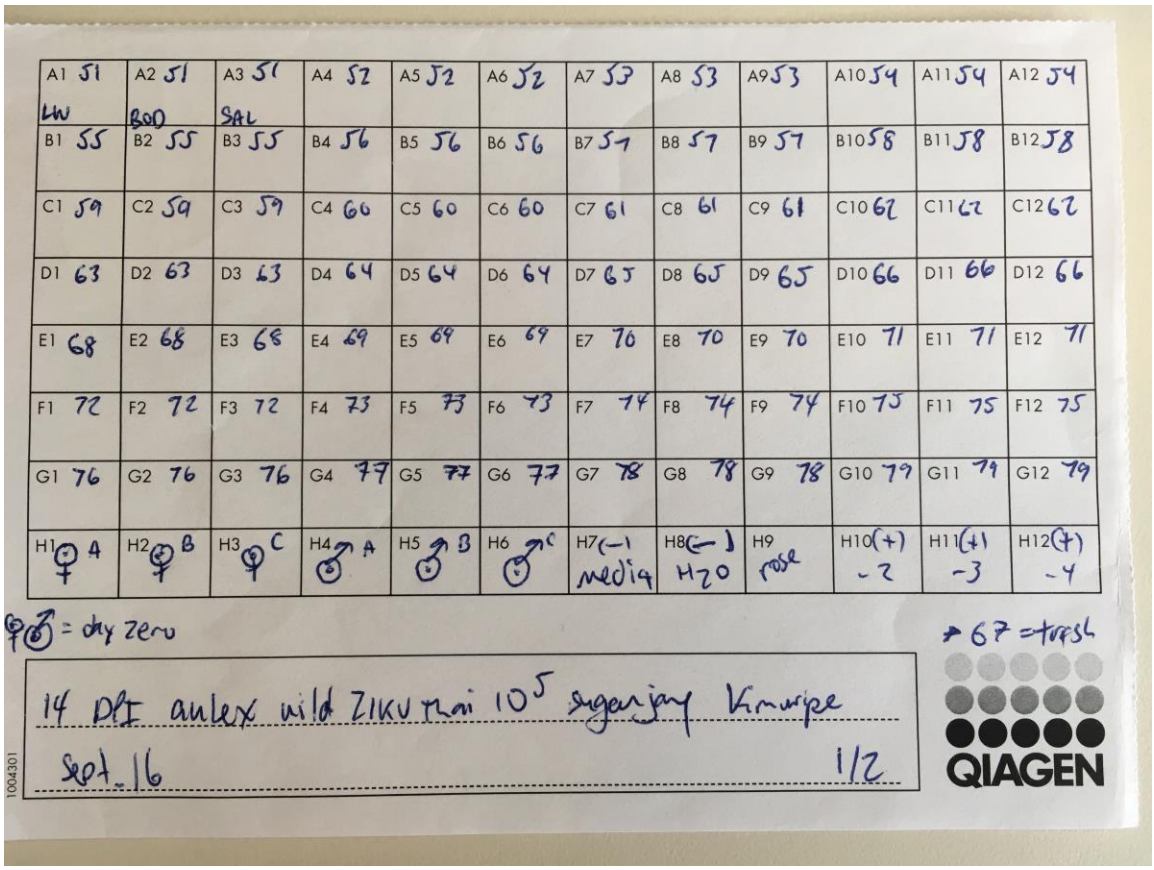
MyiQ™ BIO-RAD

PCR Quantification Report
PCR Base Line Subtracted Curve Fit Data

Current Date:	**19-Sep-16 10:56 AM**
Data generated on:	**16-Sep-16 at 10:12 PM**.
Optical data file name:	**zika 14dpi culex 2 sept 16.odm**
Plate Setup file used:	**sept15-14dpi 2.psm**
Protocol file used:	**ZIKA.tmo**
Sample volume:	**25.00 ul**
Hot Start?	**No**
Well factor collection:	**Experimental Plate**

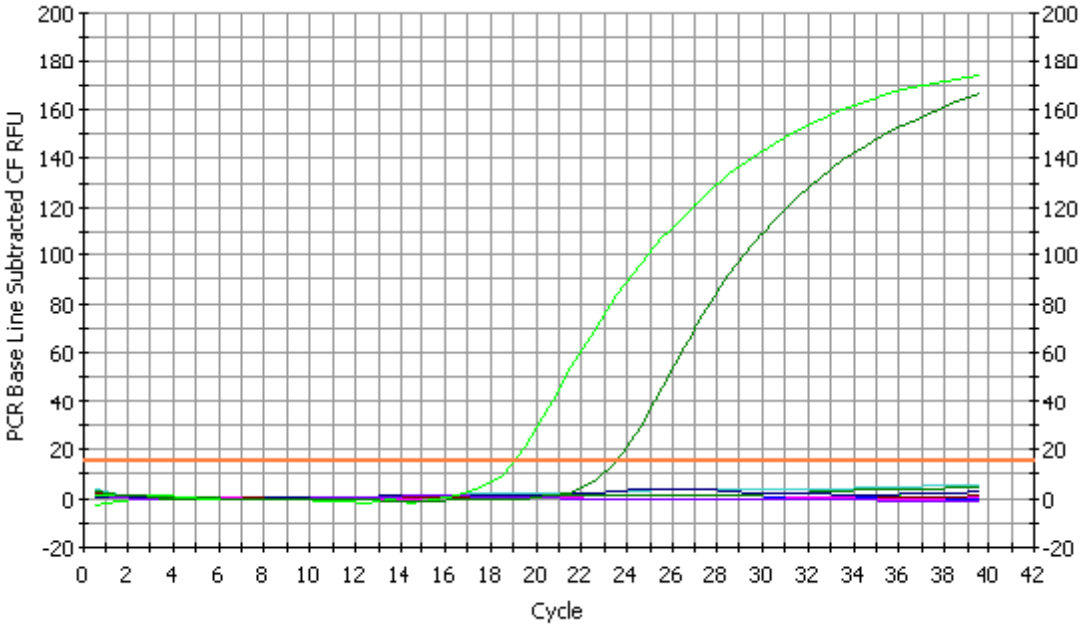
Comments

Protocol



Cycle 1: (1X)			
	Step 1:	**50.0ºC**	**for 30:00**
Cycle 2: (1X)			
	Step 1:	**95.0ºC**	**for 15:00**
Cycle 3: (40X)			
	Step 1:	**94.0ºC**	**for 00:15**
	Step 2:	**60.0ºC**	**for 01:00**
	Data collection and real-time analysis enabled.		
Cycle 4: (1X)			
	Step 1:	**4.0ºC**	**HOLD**

PCR Amp/Cycle Graph for FAM-490



Data Analysis Parameters

Calculated threshold using the **maximum curvature approach** is **15.3**.
Per-well baseline cycles have been determined automatically.
Data analysis window is set at **95.00%** of a cycle, centered at **end** of the cycle.
Weighted Mean digital filtering has been applied. Global filtering is **off**.

PCR Quantification Spreadsheet Data for FAM-490

Well	Identifier	Ct	Setpoint
A02		N/A	
A03		N/A	
A04		N/A	
A05		N/A	
A06		N/A	
A07		N/A	
A08		N/A	
A09		N/A	

H08	19.03
H09	23.54

Wells Excluded from Analysis

A total of 86 well(s) have been excluded from analysis.

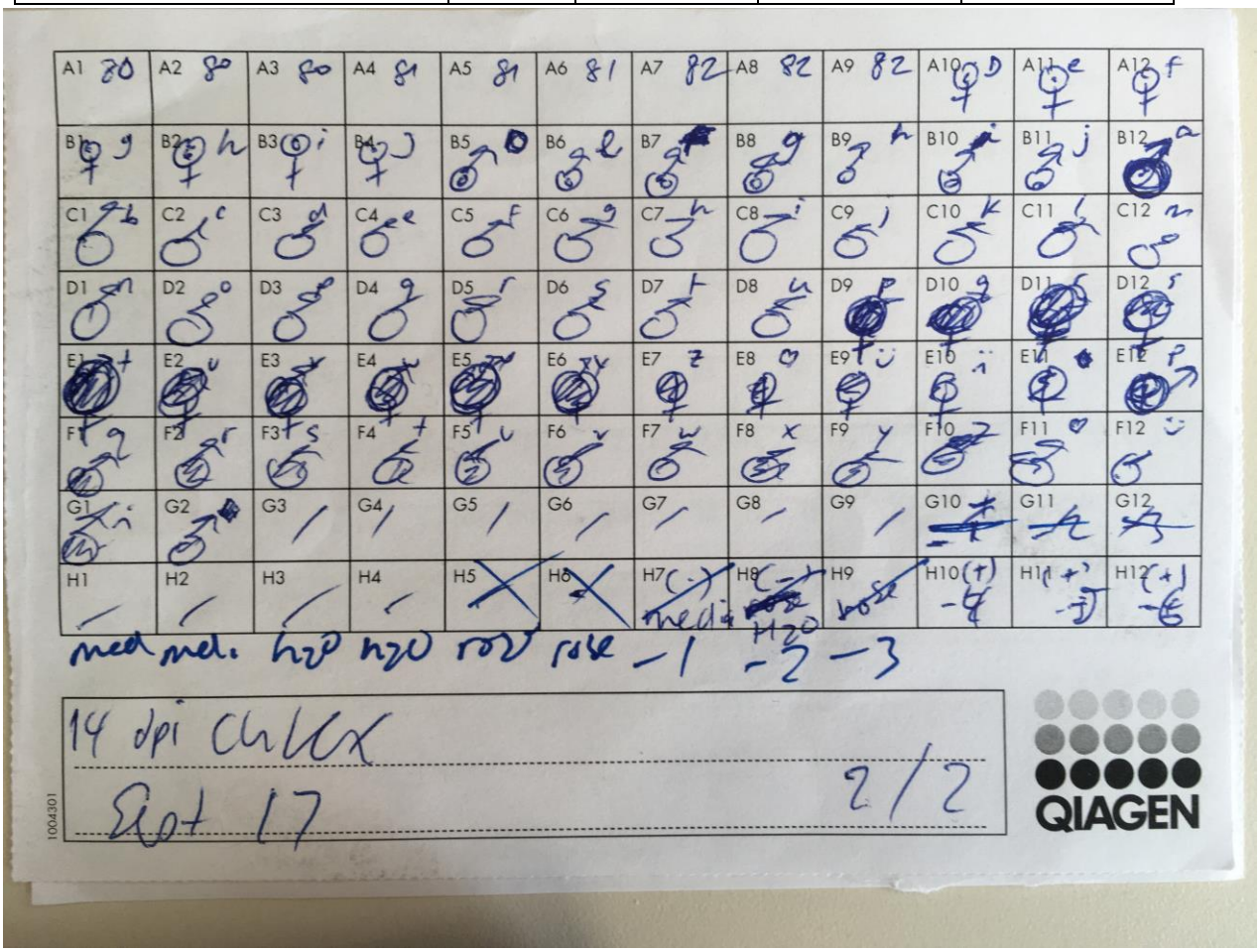
A01: <no identifier>	**A10: <no identifier>**	**A11: <no identifier>**
A12: <no identifier>	**B01: <no identifier>**	**B02: <no identifier>**
B03: <no identifier>	**B04: <no identifier>**	**B05: <no identifier>**
B06: <no identifier>	**B07: <no identifier>**	**B08: <no identifier>**
B09: <no identifier>	**B10: <no identifier>**	**B11: <no identifier>**
B12: <no identifier>	**C01: <no identifier>**	**C02: <no identifier>**
C03: <no identifier>	**C04: <no identifier>**	**C05: <no identifier>**
C06: <no identifier>	**C07: <no identifier>**	**C08: <no identifier>**
C09: <no identifier>	**C10: <no identifier>**	**C11: <no identifier>**
C12: <no identifier>	**D01: <no identifier>**	**D02: <no identifier>**
D03: <no identifier>	**D04: <no identifier>**	**D05: <no identifier>**
D06: <no identifier>	**D07: <no identifier>**	**D08: <no identifier>**
D09: <no identifier>	**D10: <no identifier>**	**D11: <no identifier>**
D12: <no identifier>	**E01: <no identifier>**	**E02: <no identifier>**
E03: <no identifier>	**E04: <no identifier>**	**E05: <no identifier>**
E06: <no identifier>	**E07: <no identifier>**	**E08: <no identifier>**
E09: <no identifier>	**E10: <no identifier>**	**E11: <no identifier>**
E12: <no identifier>	**F01: <no identifier>**	**F02: <no identifier>**
F03: <no identifier>	**F04: <no identifier>**	**F05: <no identifier>**
F06: <no identifier>	**F07: <no identifier>**	**F08: <no identifier>**
F09: <no identifier>	**F10: <no identifier>**	**F11: <no identifier>**
F12: <no identifier>	**G01: <no identifier>**	**G02: <no identifier>**
G03: <no identifier>	**G04: <no identifier>**	**G05: <no identifier>**
G06: <no identifier>	**G07: <no identifier>**	**G08: <no identifier>**
G09: <no identifier>	**G10: <no identifier>**	**G11: <no identifier>**
G12: <no identifier>	**H01: <no identifier>**	**H02: <no identifier>**
H03: <no identifier>	**H04: <no identifier>**	**H05: <no identifier>**
H06: <no identifier>	**H07: <no identifier>**	**H10: <no identifier>**
H11: <no identifier>	**H12: <no identifier>**	

Modified Well Contents

No wells have been modified.

Table 2: *Cx. pipiens* qRT-PCR Ct- values for positive ZIKV samples after 10 d.p.i. Samples highlighted in red indicate potential positive.

Cx. pipiens (10 d.p.i.)				
	Sample		Ct Value	
		Body	Leg/ Wings	Saliva
	1	N/A	N/A	N/A
	2	N/A	N/A	N/A
	3	N/A	N/A	N/A
	4	N/A	N/A	N/A
	5	N/A	N/A	N/A
	6	N/A	N/A	N/A
	7	N/A	N/A	N/A
	8	N/A	N/A	N/A
	9	N/A	N/A	N/A
	10	N/A	N/A	N/A
	11	N/A	N/A	N/A
	12	N/A	N/A	N/A
	13	N/A	N/A	N/A



	14	N/A	N/A	N/A
	15	N/A	N/A	N/A
	16	N/A	N/A	N/A
	17	N/A	N/A	N/A
	18	N/A	N/A	N/A
	19	N/A	N/A	N/A
	20	N/A	N/A	N/A
	21	N/A	N/A	N/A
	22	N/A	N/A	N/A
	23	N/A	N/A	N/A
	24	N/A	N/A	N/A
	25	N/A	N/A	N/A
	26	N/A	N/A	N/A
	27	N/A	N/A	38.01
	28	N/A	N/A	N/A
	29	N/A	N/A	N/A
	30	N/A	N/A	N/A
	31	N/A	36.01	35.13
	32	27.37	N/A	N/A
	33	N/A	N/A	35.54
	34	N/A	N/A	N/A
	35	N/A	N/A	N/A
	36	N/A	35.22	36.19
	37	35.07	N/A	37.24
	38	N/A	N/A	N/A
	39	N/A	37.42	N/A
	40	N/A	N/A	37.51
	41	N/A	N/A	N/A
	42	36.7	N/A	37.08
	43	N/A	36.99	N/A
	44	34.99	N/A	35.86
	45	N/A	36.28	N/A
	46	N/A	N/A	N/A
	47	N/A	N/A	N/A
	48	34.56	37.71	34.49
	49	N/A	35.67	N/A
	50	N/A	N/A	N/A

Table 3: *Cx. pipiens* qRT-PCR Ct- values for positive ZIKV samples after 14 d.p.i. Sample highlighted in red indicate potential positive.

Culex pipiens (14 d.p.i.)	1	N/A	N/A	N/A
	2	N/A	N/A	N/A
	3	N/A	N/A	N/A
	4	N/A	N/A	N/A
	5	N/A	N/A	N/A
	6	37.08	N/A	N/A
	7	N/A	N/A	N/A
	8	N/A	38.25	37.38
	9	N/A	N/A	N/A
	10	N/A	N/A	N/A
	11	N/A	N/A	N/A
	12	N/A	N/A	N/A
	13	N/A	N/A	N/A
	14	N/A	N/A	N/A
	15	N/A	N/A	N/A
	16	N/A	N/A	N/A
	17	N/A	N/A	N/A
	18	N/A	N/A	N/A
	19	N/A	N/A	N/A
	20	37.39	N/A	N/A
	21	N/A	N/A	N/A
	22	N/A	N/A	N/A
	23	N/A	N/A	N/A
	24	N/A	N/A	N/A
	25	N/A	N/A	N/A
	26	N/A	N/A	37.69
	27	N/A	N/A	N/A
	28	N/A	N/A	N/A
	29	N/A	N/A	N/A
	30	N/A	N/A	N/A
	31	N/A	N/A	N/A
	32	N/A	N/A	N/A